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In situ analyses of inflammatory colitis in Muc2$^{-/-}$ mice

(diploma thesis)

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In situ analýzy zánětlivých kolitid Muc2$^{-/-}$ myší

(diplomová práce)

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V Hradci Králové Martina Drábková

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CONTENT

ABSTRACT

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Title: *In situ* analyses of inflammatory colitis in Muc2$^{-/-}$ mice
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Background: Muc2 mucin is the main structural component of the intestinal mucus which forms important protective barrier avoiding the direct contact between the commensal bacteria and the epithelium. In order to understand the role of Muc2 mucin a Muc2 deficient mice strain has been developed. Initial observation showed that these mice already in few weeks after the birth develop spontaneous colitis and later on colon cancer. To better understanding what is going on in the colon and small intestine of Muc2$^{-/-}$ mice, in this thesis we focused on *in situ* analyses of distribution of different immune cells in these tissues.

Methods: In our experiments three Muc2$^{-/-}$, two Muc2$^{+/-}$ and three wild type mice on the C57BL/6 background were used. For the detection of distribution of different immune cells within lamina propria of small intestine and colon the immunohistochemistry analyses on sections from these tissues were performed.

Results: In the lamina propria of distal colon of Muc2$^{-/-}$ mice we found an increase of neutrophils followed by increase of macrophages, CD4^{+} T cells and B cells/plasma cells compared to control and wild type animals. In the proximal colon there was a significant increase of neutrophils in the most inflamed mice and slightly higher amount of macrophages and CD4^{+} T cells. The number of DCs was in both distal and proximal colon unaltered. In the small intestine we did not observe any changes with the exception of B cells. The number of B cells/plasma cells scattered in the LP of small intestine from Muc2$^{-/-}$ mice was significantly higher compared to control and WT mice.

Conclusions: Our results have shown that the loss of the mucus leads to the spontaneous inflammation of colon which is more severe in the distal part. The inflammation is followed by massive increase of inflammatory immune cells, neutrophils and macrophages, and later on leads to activation of specific immune response followed by increase of CD4^{+} T cells and B cells/plasma cells.

ABSTRAKT

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Cíl práce: Muc2 mucin je hlavní strukturální součástí hlenu pokrývající střevní sliznici. Ten tvoří významnou ochranou bariéru zabraňující přímému kontaktu střevních bakterií se střevním epitelem. Pro lepší studium role Muc2 mucinu byl vyvinut Muc2 deficientní myší kmen. První studie ukázaly, že u těchto myší dochází již během několika týdnů po narození ke spontánnímu vzniku střevního zánětu, který může později vést až k rozvoji kolorektálního karcinomu. Pro lepší pochopení změn v tenkém a tlustém střevě Muc2$^{-/-}$ myší jsme se v této práci zaměřili na *in situ* analýzy distribuce různých imunitních buněk v těchto tkáních.

Metody: Do našich experimentů jsme zahrnuli tři Muc2$^{-/-}$, dvě Muc2$^{+/-}$ a tři wild type (standardní) myši z C57BL/6 kmenu. Distribuce imunitních buněk v lamina propria tenkého a tlustého střeva těchto myší byla detekována metodou imunohistochemického barvení na jednotlivých tkáňových řezech.

Výsledky: V lamina propria distální části tlustého střeva Muc2$^{-/-}$ myší jsme zjistili nárůst počtu neutrofilů společně s vzrůstem množství makrofágů, CD4^{+} T buněk a B buněk/plazmatických buněk v porovnání s kontrolními zvířaty. V proximální části tlustého střeva byl nárůst neutrofilů patrný zvláště u myší s nejintenzivněji probíhajícím zánětem. Množství makrofágů a CD4^{+} T buněk bylo zvýšeno mírně. Množství dendritických buněk v proximální i distální části tlustého střeva zůstalo nezměněno. V tenkém střevě jsme nepozorovali žádné změny, s výjimkou nárůstu množství B buněk/plazmatických buněk v lamina propria tenkého střeva u Muc2$^{-/-}$ myší v porovnání s kontrolními zvířaty.

Závěry: Naše výsledky ukazují, že ztráta hlenu kryjícího střevní sliznici vede k rozvoji spontánního zánětu tlustého střeva, který je lokalizován zvláště v distální oblasti. Zánět je doprovázen nárůstem počtu zánětlivých imunitních buněk - neutrofilů a makrofágů - a později vede k aktivaci specifické imunitní odpovědi doprovázené nárůstem počtu CD4^{+} T buněk a B buněk/plazmatických buněk.

ABBREVIATIONS

Ab	antibody
APC	antigen presenting cells
DCs	dendritic cells
DSS	dextran sodium sulphate
GALT	gut associated lymphoid tissues
GIT	gastrointestinal tract
H&E	hematoxylin and eosin
IBD	inflammatory bowel disease
Ig	immunoglobulin
IL	interleukin
LP	lamina propria
MLN	mesenteric lymph nodes
M cells	membrane cells
$Muc2^{-/-}$	Muc2 knockout mice
$Muc2^{+/-}$	Muc2 heterozygous mice
Neu	neutrophils
PPs	Peyer´s patches
PRRs	pattern recognition receptors
Th cells	T helper cells
TLR	Toll-like receptors
Treg	regulatory T lymphocytes
UC	ulcerative colitis
WT	wild type

1 Introduction

The mammalian intestine is colonised with a very dense and diverse population of bacteria. Commensal bacteria live in a symbiotic relationship with its host and have a several essential contribution to the human physiology and health. They are crucial for digestion of dietary polysaccharides, development of the mucosal immune system; moreover they compete with potential bacterial pathogens for space and nutrients and thus play important role in the protection against pathogenic infection.

In order to maintain homeostasis in the gut there are many different barriers and mechanisms protecting the host against undesired intestinal inflammation caused by commensals. One of these mechanisms is a secretion of protective mucus which covers the epithelial cell layer. In this project we focused on the role of the secreted gel-forming mucin Muc2, which is the main structural component of intestinal mucus.

A Muc2 deficient ($Muc2^{-/-}$) mice strain which lack the gene for Muc2 mucin has been developed. Lack of the main structural component of mucus results in general loss of the protective inner mucus layer, which under normal, healthy condition avoids direct contact of commensal bacteria with the epithelium. Initial observation showed that these mice develop spontaneous colitis and colon cancer. In different studies it was shown that bacteria in colon of $Muc2^{-/-}$ mice get in direct contact with the epithelial cell layer.

Initial studies in our group have focused on the composition of cells isolated from lamina propria from colon and small intestine of $Muc2^{-/-}$ mice. It was shown that once the inflammation begins $Muc2^{-/-}$ mice have higher ratios of neutrophils compared to Muc2 heterozygous ($Muc2^{+/-}$), referred as controls, or wild type animals and the number of neutrophils is higher in the distal part of colon. Furthermore it was discovered that in tissues of inflamed $Muc2^{-/-}$ mice the up-regulation of pro-inflammatory cytokines is not significant and instead of that anti-inflammatory cytokines are decreased compared to control animals. This may indicate that the cause of the inflammation in these animals is rather the loss of tolerance than an activated immune response.

2 Aim and task of the thesis

The aim of this thesis is to give better overview on the immune status of the mucosal compartment of small intestine and colon of $Muc2^{-/-}$ mice as well as control mice. Therefore in this study we focus on the *in situ* situation in tissues of interest. We want to exanimate the cellular composition of different immune cell types in these tissues using an immunohistochemistry method.

Next aim is to compare distribution of immune cells in proximal and distal colon to confirm the hypothesis that the distal part of colon is more affected by the inflammation.

3 Theoretical background

3.1 Histology and anatomy of the intestine

Intestine is a tubular organ specialised for digestion and absorption of nutrients. Throughout its length the wall of the intestine is made of three principle layers:

- mucosa with submucosa
- muscularis
- serosa

Mucosa is lined with an epithelial layer and has fibrovascular stroma called the lamina propria (LP). Submucosa is built from connective tissue surrounding blood and lymph vessels and nerves and is separated from the LP by the thin layer of smooth muscle, so-called lamina muscularis mucosae. Muscularis is the middle layer made of outer longitudinal and inner circular layer of smooth muscle and is covered by the thin layer of visceral peritoneum – the serosa.

The epithelium is made by many types of specialised cells. Absorptive cells (enterocytes) with the luminal cell membrane forming microvilli are the most common cell type of the mucosal epithelium. Between them mucous goblet cells are scattered. Goblet cells are specialised for the mucus secretion. Another type of epithelial cells is Paneth cells. Paneth cells occur in the small intestine (mainly in jejunum) and they contain in its cytoplasm granules with lysozyme and antimicrobial peptides. These granules are especially large in the mouse. Enteroendocrine cells are polypeptide producing endocrine cells which are diffusely distributed along gastrointestinal tract (GIT).

Intestine consists of two main parts - small intestine and large intestine. Small intestine is subdivided into duodenum, which is the place of pancreatic and bile secretion, jejunum and ileum. This organ is highly efficient in digestion and absorption of nutrients. In order to enlarge the absorptive surface the mucosa forms finger like projection into the intestinal lumen so-called villi, consisting of epithelium and LP, and protrusions in opposite direction beneath the mucosal surface – crypts. Mucosal surface of the murine small intestine does not form folds (plicae) as we can find in the small intestine of human.

Large intestine is formed by caecum, colon and rectum. Mucous membranes contain larger proportion of goblet cells compared to small intestine and forms crypts

but not villi. In the colon the ascending, transverse and descending part is described. Mucosa of ascending and transverse colon forms transverse folds, whereas the descending part and rectum have longitudinal folds formed by mucosa and submucosa which protrude into the lumen (Hedrich, Bullock, 2004).

3.2 Intestinal microflora

The intestinal tract with the surface covering an area of approximately 100 m^2 is the largest mucosal surface of the human body. The mucosal surface of the intestine is in direct contact with the external environment and therefore is susceptible to colonization by microorganism (Artis, 2008). The mammalian intestine is colonised mainly by bacteria, which enter our body already shortly after the birth. The density and diversity of so-called commensal bacteria increase along the GIT from the stomach to the colon. In the colon the density of commensal bacteria can reach 10^{12} of more than 400 distinct species of organism per gram of intestinal content (Macpherson, Harris, 2004).

Comparisons between experimental germ-free animals and those containing normal bacterial microflora have shown that the mucosal immune system is highly adapted to the presence of commensals and during millions of years of co-evolution a symbiotic relationship between commensal bacteria and host was established. Commensal bacteria have several essential contributions to the human physiology and health. They are crucial for degrading of dietary polysaccharides; they contribute to the development of the intestine and the mucosal immune system, including epithelial cell maturation, angiogenesis and lymphocyte development. Moreover, commensal bacteria play important role in protecting their host against pathogenic infection by competing with bacterial pathogens for space and dietary nutrients. In return benefit bacteria from the nutrient-rich environment in the host intestine (Hooper, Macpherson, 2010).

However this symbiotic relationship between bacteria and host can be disturbed. Disruption of the balance between tolerance and immunity can contribute to pathologies such as food allergy, inflammatory bowel disease (IBD) or intestinal cancer (Artis, 2008).

3.3 Mucosal immune system

Mucosal epithelia form the barrier between the internal and external environment and therefore are important site of entry for many microorganism. The mucosa-associated lymphoid tissues represent the most abundant lymphoid structure in the human body and in the gut are known as gut-associated lymphoid tissue (GALT). GALT can be functionally divided into inductive and effector sites.

The inductive sites of GALT include mesenteric lymph nodes (MLN), Peyer´s patches (PPs) of the small intestine, colonic patches and isolated lymphoid follicles. These are the places where the immune response is induced (Newberry, Lorenz, 2005).

PPs are organised lymphoid tissues present only in the small intestine. In the central region there are B lymphocytes (B cells) - rich areas often containing germinal centres. Small number of T lymphocytes (T cells), mainly $CD4^+$ T cells, is scattered interfolliculary. The overlaying epithelial layer contains specialised M (membrane) cells. M cells lack microvilli and actively take up macromolecules from the intestinal lumen into subepithelial tissues by pinocytosis. At the basal surface underlying lymphocytes and antigen-presenting cells (APC) take up the transported material and process it for antigen presentation (Abbas, Lichtman, 2005).

The effector sites of GALT represent epithelium and underlying LP (Newberry, Lorenz, 2005). Epithelium contains intraepithelial lymphocytes which monitor epithelial damage and might recognise microbial antigens (Cheroutre, 2004). LP contains mix population of cells: T cells – most of which are $CD4^+$ T cells, large number of activated B cells and plasma cells, macrophages, dendritic cells (DCs), eosinophil and mast cells (Abbas, Lichtman, 2005).

In order to maintain homeostasis the mucosal immune responses has to be tightly regulated. The intestinal immune system has to remain immunologically hyporesponsive to the commensal bacteria and food antigens avoiding potentially harmful overreaction and on the other hand maintain the ability to response to a pathogenic challenge with an appropriate degree of inflammation (Hooper, Macpherson, 2010).

3.4 Cells of the immune system

Cells of the immune system originate from the pluripotent hematopoietic stem cells in the bone marrow. These pluripotent cells give arise more specific stem cells – a common myeloid progenitor and a common lymphoid progenitor. The myeloid progenitor is the precursor of different types of leukocytes - macrophages, granulocytes, DCs and mast cell. All these leukocytes play crucial role in the innate immunity. Beside this myeloid progenitor gives rise to other important blood elements - erythrocytes and megakaryocytes. The lymphoid progenitor is precursor of the lymphocytes and natural killers and is also able to give rise to DCs (Janeway, 2005).

3.4.1 Macrophages

Macrophages are the mature form of monocytes. Monocytes are incompletely differentiated; they circulate in the blood and differentiate into macrophages first after entering the tissue. Macrophages are distributed in all organs and connective tissues where they play important role in the both innate and adaptive immunity. Their function in the innate immunity is primarily phagocytic. They engulf microbes and produce cytokines that recruit and activate other inflammatory cells. Macrophages containing ingested microbes can also display microbial antigens on their surface where it could be recognised by T cells. Therefore they may also act as an APC (Abbas, Lichtman, 2005).

One of the largest populations of macrophages in the body is found in the healthy intestinal mucosa. In both, small and large intestine, they are located in the LP in close contact with the epithelium, in the small intestine they also appear in the sub-epithelial dome region of PPs. Subepithelial macrophages rapidly phagocyte penetrant bacteria. After that they kill ingested microorganism through mechanism that include antimicrobial proteins and reactive oxygen species (Kelsall, 2008).

Resident macrophages in the intestine are distinct in their functional properties from other macrophages populations in the body. Unlike macrophages from many other tissue sites intestinal macrophages are normally relatively non-inflammatory with lower expression of bacterial receptor molecules. In addition, they do not produce pro-inflammatory cytokines, including interleukin-1 (IL-1), IL-6 or tumor necrosis factor α (TNFα), and only low levels of IL-8 following bacterial recognition. While their phagocytic and bactericidal functions retain efficient. Thus it seems that intestinal

macrophages are in the state of partial activation in which they are able to clear penetrated bacteria without provoking inflammation (Smythies et al., 2005; Platt, Mower, 2008). It is believed that this could be an evolutionary adaptation which prevents potentially damaging from being activated under normal, homeostatic conditions (Hooper, Macpherson, 2010).

3.4.2 Dendritic cells

Dendritic cells (DCs) are immune cells characterised by cytoplasmic projection - dendrites. They are located in places where the most of the microbes can enter: the epithelia of the skin, GIT and respiratory systems. These cells are so-called professional APC. That means they present antigens to T cells. This process is very important for the activation of the adaptive immune response.

Immature cells migrate from the blood to reside in the tissue, where they capture and process antigens. DCs take up extracellular antigens either non-specifically – by macropinocytosis or they are able to recognise and ingest pathogens through receptors, which recognize features common to microbial surfaces, so-called pattern recognition receptors (PRRs). One of the most important groups of these receptors is group of Toll-like receptors (TLR). After encountering antigen DCs rapidly mature and migrate to lymph nodes, where they display the antigen to naive T cells. This results in in their activation, clonal expansion and differentiation (Janeway, 2005).

Intestinal DCs are located in the intestinal PPs and lymphoid follicles. In PPs DCs sample antigens which were translocated through M cells. They are also scattered in the LP, where they continually sample their local environment. In LP processes of some DCs can penetrate through tight junctions of the intestinal epithelium and sample the microorganism from the mucus layer or the lumen. Moreover they are present in MLN, where they continually migrate after encountering antigen (Westendorf et al., 2010).

Intestinal DCs play crucial role in balancing tolerance to self-antigens with immunity to pathogens. When loaded with pathogen, DCs migrate to PPs and MLN and express high level of major histocompatibility complex class I and II as well as co-stimulatory molecules, which provide the second signal to T cells initiating adaptive immune responses. On the other hand, when they are loaded with food or self-antigen the expression of co-stimulation molecules is missing and instead of inflammation

the tolerance is induced (Janeway, 2005). The commensal-loaded DCs that migrated to the MLN do not penetrate further to the systemic immune system and are restricted to the mucosal immune compartment. Therefore the immune response against commensal bacteria is induced only locally without inducing unnecessary systemic immunity (Macpherson, Uhr, 2004).

Mucosal DCs also differ from the other DCs in the human body as the local environment has a big influence on their phenotype and function. It is a heterogeneous population, which could be subdivided depending on the surface markers expression. The different subsets have different function and properties (Strauch et al., 2010).

In contrast to splenic DCs large proportion of intestinal DCs expresses surface marker CD103 (Hooper, Macpherson, 2010). These DCs when activated by antigen promote the development of Treg response. Moreover it was shown that intestinal DCs produce anti-inflammatory cytokines and do not release significant amounts of pro-inflammatory mediators after stimulation (Sato at al., 2003; Strauch et al., 2010). They also express lower level of TLR and are less responsive to microbial-derived TLR ligands compared to spleen or blood-derived DCs (Takenaka et al., 2007). Thus under steady-state condition mucosal DCs induce a non-inflammatory environment to avoid inflammatory reaction in response to commensals (Westerndorf et al., 2010).

Furthermore DCs containing commensal bacteria selectively induce B cells to differentiate into plasma cells producing immunoglobulin (Ig) A specific for intestinal bacteria. Plasma cells translocate from lymphoid sites into the intestinal LP and secrete IgA, which is transcytosed across the epithelial cell layer and binds to bacteria on the luminal side of epithelium. This helps to protect against mucosal penetration by commensal bacteria (Macpherson, Uhr, 2004).

3.4.3 Neutrophils

When the pathogens breach the epithelial barrier macrophages located subepithelial are the first cells to encounter them. Activated macrophages release chemokoines (chemoattractant cytokines) that attract other inflammatory cells and soon they are reinforced by neutrophils (Neu). Alike macrophages Neu are phagocytic cells important for innate immunity. They are able to recognize, ingest and destroy many pathogens without the aid of adaptive immune response. They circulate in the blood, but they are not present in normal, healthy tissue. Neu enter the tissue only at sites of

inflammation. These cells are relatively short lived and their number increase rapidly during immune responses (Janeway, 2005).

3.4.4 Lymphocytes

Lymphocytes – cells of the adaptive immune system - are the only antigen specific immune cells. They are unique in being able to mount a specific immune response against virtually any foreign antigen. On its cell-surface they bear antigen-specific receptors, whereas every individual lymphocyte carries receptors of a single specificity so that the total repertoire of receptors is virtually able to recognise any pathogen.

There are two major type of lymphocyte: T lymphocytes (T cells) which mature in thymus and B lymphocytes (B cells) which mature in bone marrow. Mature cells continually circulate between blood and peripheral lymphoid tissues. After encountering with antigen B cells differentiate into antibody-secreting plasma cells while naive T cells differentiate into effector T cells. Moreover, both T and B cells may differentiate into memory cells which are ready to response rapidly and more effective on a second encounter with antigen (Jenaway, 2005).

The two main classes of effector T cells are cytotoxic T cells or $CD8^+$ T cells, which kills cells infected by viruses and T helper cells or $CD4^+$ T cells. (Janeway, 2005) In response to cytokine induced signals $CD4^+$ T cells differentiate in more specific subsets: T helper 1 subset (TH1), which activate macrophages and B cells during the immune response against intracellular pathogens; T helper 2 cells (TH2), which activate mast cells, eosinophils and increase production of IgE during the host defence against helminthic parasites and T helper 17 (TH17), which mediate immune response against extracellular bacteria and fungi. Additionally there are two classes of $CD4^+$ regulatory cells (Treg), which when activated by antigen suppress effector responses. The balance between the function of Treg and $CD4^+$ effector cells is crucial for the mucosal homeostasis (Westendorf et al., 2010; Hooper, Macpherson, 2010).

3.5 Intestinal epithelial cell layer

There are numerous physical adaptations to prevent penetrating of microorganism beneath the epithelium. The important barrier between the intestinal lumen and host connective tissues forms the epithelial cell layer. This is a single cell layer where epithelial cells are joined firmly together by tight junctions avoiding penetration of pathogens and large molecules (Madara, Nash, Moore, 1990). In order to minimize contact between luminal microorganism and the epithelial cell surface this physical barrier is reinforced by a continuous layer covering the apical side of intestinal epithelial cells composed of mucus and the glycocalyx (Mafalhaes, 2007).

In addition to physical barrier function epithelium takes an active part in inducing the innate and adaptive immune systems. Epithelial cells directly sense commensals and pathogens through recognition by PRRs, such as membrane bound TLR or cytosolic Nod-like receptors (NLR). These receptors specifically recognise the molecular features common for many microorganism so-called pathogen-associated molecular patterns (PAMPs). The interaction of PRRs with PAMPs induces different signalling pathways and, in result, triggers the immune response. These receptors are present on the apical cell-surface but most of them are expressed only on the basolateral surfaces or in the cytoplasm (NLR) of epithelial cells. The intracellular and basolateral distribution of PRRs suggest one mechanism by which intestinal epithelial cells retain the capacity to respond to pathogens while maintaining the tolerance to commensal bacteria (Philpott, Girardin, 2004).

Bacterial pathogens differ from non-pathogenic and commensal bacteria by the presence of specific pathogenicity genes, which are often organised in so-called pathogenicity islands acquired during evolution via horizontal genetic transfer (Hacker, Carniel, 2001). These genes encode adherence factors, toxin, invasin and other virulence factors, which allow pathogens to invade the epithelial cells layer and infect the host (Schmidt, Hensel, 2004). In contrast, commensal bacteria lack pathogenicity islands, they do not invade the epithelium and thus can´t be recognised by basolateral and intracellular PRRs.

Epithelial cells also secrete a broad range of antimicrobial proteins including defensins, cathelicidins and C-type lectins, which kill bacteria either directly through enzymatic attack of the bacteria cell or by disrupting the bacteria inner membrane. These proteins retain in the mucus layer and do not penetrate to the luminal content.

Several of these proteins are expressed constitutively but the expression of a key subset of antimicrobial proteins is controlled by bacterial signals through activation of PPR. (Hooper, Macpherson, 2010)

Antimicrobial protein can be produced by virtually all epithelial cell lineages. However, in small intestine there is a specialize lineage of secretory cells - Paneth cells. Paneth cells reside at the base of crypts and directly sense enteric bacteria triggering expression of diverse repertoire of antimicrobial proteins and thus limit penetration of host tissues by bacteria that localize at the mucosal surface, in or beneath the mucus layer (Vaishnava et al., 2008). By secretion of antimicrobial proteins and sensing bacteria and bacterial antigens Paneth cells contribute to the intestinal innate immunity (Aybe et al., 2000).

3.6 The mucus layer

Mucus has an important role in the protection of the GIT. The main components of the mucus are the gel-forming mucins, which form complex, large and gel-like structures. Mucins are large highly glycosylated proteins characterised by long repetitive domains rich in amino acid serine, threonine and proline (so-called PTS domain). Free hydroxyl groups on amino acids in this PTS domain are frequently heavy O-glycosylated with different types of glycans. The major and the most important structure component of the mucus layer is a Muc2 mucin (Johansson, Thomsson, Hansson, 2009).

The colonic mucus consists of two layers with the same composition but different properties. The inner layer is densely packed with a high concentration of Muc2 mucin and is firmly attached to the epithelial surface. A gradient in increasing thickness of this layer along the colon was observed. The thickness of the inner mucus layer in the distal colon of mouse is approximately 50μm. This inner layer is devoid of bacteria and builds an important barrier, which separates bacteria from the intestinal epithelia.

Outer layer is loose, easily removed and despite inner layer is colonised by bacteria. Vast number of glycans provides a good energy source and attachment place for commensal bacteria and thus serves as a good habitat for them. Outer layer is estimated to be twice as thick as the inner layer, but the thickness is variable as it can be degraded by the commensal flora and transported distally together with the luminal

content. The almost identical composition of the loose and firm mucus layer suggest that the loose layer is generated from the firm mucus layer (Johansson et al. 2008). In contrast in small intestine the Muc2 mucin directly forms a soluble mucus layer and the mucus does not form continuous mucus layer (Johansson, Larsson, Hansson, 2011).

3.7 Mucus turnover

Mucus is secreted by the goblet cells which differentiate from the crypt stem cells in the crypt bottom. During the maturation goblet cells migrate from the crypt bottom towards the crypt opening and they fill their granules with Muc2 mucin. Mature goblet cells secrete their content mostly at the upper part of the crypt or at the bottom of the villus in the small intestine. Muc2 mucin is stored in the goblet cell in a condensed way and during the release expands dramatically in volume and forms the polymeric net-like mucus layer that is firmly attached to the epithelium. The secretion of Muc2 mucin is continuously refilling the inner mucus layer from underneath. At the certain distance from the epithelium this firmly attached layer is converted into the loose outer mucus layer. This happens probably due to proteolytic cleavage in the cysteine-rich parts of Muc2. These cleavages allow mucus to expand approximately four times in volume and form the outer mucus layer. The outer mucus layer is less organised and degraded by the bacteria, which bind to Muc2 mucin and use glycan as an energy source. Finally part of this loose layer is passed distally with the luminal content (Johansson, Larsson, Hansson, 2011).

The total turnover of the mucus is faster in colon than in small intestine. The production and secretion of new mucus in distal colon can take less than 5 hours. Small amount of mucus is secreted constantly on the basal level however the massive secretion is trigged be the stimulation. There is a variety of stimuli, which can trigger the secretion, for example cholinergic agonists, neuropeptides, nucleotides, hormones, immune system mediators, nitric oxide as well as bacterial products (Johansson, 2009).

3.8 Inflammatory bowel disease and role of the mucus

Ulcerative colitis (UC) together with Crohn's disease are two forms of chronic inflammatory bowel disease (IBD) characterised by chronic inflammation of the GIT. These two main forms have many similarities, but there are also many differences in clinical and pathological characteristics. In Crohn's disease any part of GIT can be affected, but mostly it is ileum, colon, peri-anal area and caecum. Characteristic for this form of IBD is the presence of unaffected parts of bowel between affected regions. In UC the inflammatory process involves rectum and extends continuously proximally but remains restricted to the colon (Bouma, Strober, 2003).

So far the pathogenesis of this disease had not been totally clarified. However, it is believed that it´s caused by multiple environmental and genetic factors involving the immune system, microbial factors and the intestinal epithelial barrier. One of the critical factors in the manifestation of IBD is an existence of an immune imbalance.

It was shown that in active UC Muc2 synthesis and secretion is decreased (Tytgart et al., 1996) and the colonic mucus layer is thinner (Pullan et al., 1994). Such alteration in the protective functions of the intestinal mucus may result in an increased association of luminal commensal bacteria with the epithelium and trigger or enhance the inflammatory process during UC. To directly address whether Muc2 is involved in epithelial protection and pathogenesis of inflammatory colitis $Muc2^{-/-}$ mice were generated through the genetic inactivation of murine Muc2 gene (Velcich, 2002).

3.9 Muc2 deficiency and $Muc2^{-/-}$ mice as an experimental animal

Muc2 mucin is a main structure component of the colonic mucus layer and keeps this protective layer together. The lack of this mucin results in the loss of the outer and inner mucus layer. Without the inner mucus layer bacteria get in the direct contact with the epithelial cell layer and may easily penetrate beneath the epithelium (Johansson et al., 2008). This contributes to the onset of experimental colitis in $Muc2^{-/-}$ mice. These mice spontaneously develop a mild colitis when they are colonised by normal enteric bacteria. Yet they are extremely susceptible to cytotoxic luminal agents like dextran sodium sulphate (DSS). The treatment with DSS led to very severe colitis within few days (Johansson et al., 2010).

Loss of Muc2 in this experimental models leads to breach of the epithelial barrier and abnormal morphology in the colon regarding an increase in thickness of the gut mucosa, flattening and ulceration of epithelial cells, general loss of crypt architecture and overall structure of the LP. Goblet cells in the colon of $Muc2^{-/-}$ animals are smaller; more condensed and lack their round bell shape. In addition a mild increase of inflammatory cells, an increase in proliferation and a decrease of cell differentiation is observed. These alterations are much more severe in distal colon compared with the proximal colon and aggravate as the mice aged.

Objectively $Muc2^{-/-}$ animals show many symptoms of intestinal inflammation as diarrhoea, occult blood loss to gross bleeding and malnutrition, which probably cause the growth retardation compared with $Muc2^{+/-}$ and WT animals (Van der Sluis et al., 2006).

4 Experimental part

4.1 Material

4.1.1 Mice

For the experiments three Muc2 knockout ($Muc2^{-/-}$), two Muc2 heterozygous ($Muc2^{+/-}$) and three wild type (WT) mice on the C57BL/6 background were used. All mice were kept at the Experimental Biomedicine animal facility at the Gothenburg University under pathogen-free condition and were provided with food and water *ad libitum*. Their medical conditions and weight were observed once a week. All experiments were performed with 20 - 25 weeks old animals of both genders in accordance with protocols approved by the government animal ethics committee.

4.1.2 Laboratory material

- Microscope slides - Superfrost PLUS (Menzel-Gläser)
- Cover slips (Menzel-Gläser)
- DAKO pen (Abcamn, UK)
- Disposable vinyl specimen molds, 15x15x15mm (Tissue-Tek®, Sakura Finetek Europe)
- Cryostat – Leica
- Fluorescent microscope – Carl Zeiss

Software:

- AxioVision Rel. 4.8.1
- ImageJ
- GraphPad Prism 5

4.1.3 Chemicals

- Accustain® Eosin Y solution alcoholic (SIGMA-ALDRICH, Germany)
- Accustain® Hematoxylin solution (SIGMA-ALDRICH, Germany)
- Acetone 50%, 100% (Histolab, Sweden)
- Acid ethanol – 1ml concentrated HCl (Sharlau) + 400 ml 70% ethanol

- Avidin/Biotin blocking kit (Vector Laboratories)
- Ethanol 95%, 100% (Kemetyl, Sweden)
- Isopentane (VWR, Sweden)
- Liquid nitrogen (Air Liquid, Sweden)
- Normal goat serum (NGS) 5%, 1%
- Pertex - xylene based mounting medium – (Histolab, Sweden)
- Phosphate buffered saline (PBS)
- ProLong Gold antifade reagent with DAPI (Invitrogen Molecular Probes)
- Tissue transporting medium – Histocon (Histolab, Sweden)
- Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe)
- Xylene (VWR, Sweden)

4.1.4 Antibodies

Primary antibodies:

- Purified anti-mouse Ly-6G 1A8 0,5mg/ml (BD Pharmigen) 1:100
- Biotin anti-mouse CD45R/B220 1mg/ml (BD Pharmigen) 1:500
- Rat anti-mouse CD4 (LT3) 1,4 mg/ml (BD Biosience) 1:100
- Biotin anti-mouse CD11c (HL3) 0,5 mg/ml (BD Pharmigen) 1:100
- Rat anti-mouse F4/80 1 mg/ml (AbD Serotec, Germany) 1:100

Secondary antibodies:

- Alexa Flour 488 goat anti-rat IgG (H+L) 2mg/ml (Molecular Probes) 1:1000, 1:500, 1:400,
- Streptavidin, Alexa Fluor 594 conjugate 1mg/ml (Molecular Probes) 1:200

Isotype control:

- Purified rat IgG2α,κ 1mg/ml (NA/LE) 1:100
- Biotin mouse anti-rat IgG2α (RG7/1.30) 0,5 mg/ml (BD Pharmigen) 1:500
- Purified armenian hamster IgG isotype (HTK888) 1mg/ml (BioLegend) 1:100

4.2 Methods

4.2.1 Tissue samples

Mice were sacrificed by the cervical dislocation and the colon and small intestine (ileum) were removed and placed in tissue transporting medium. Colon was opened longitudinally and the luminal content was carefully removed and discarded. Next the colon was longitudinally divided in two pieces. One was used for preparing the "Swiss rolls" samples and the second part was used for other experiments (FACS, CBA analyses). Each segment was rolled up using a toothpick so that the distal part of the colon was in the middle of the "Swiss roll". Then, the "Swiss roll" was carefully removed from the toothpick into disposable vinyl specimen mold filled with TissueTek O.C.T. compound. The mold was closed with a cork plate. The same procedure was done with small intestine. Finally, tissue samples were snap frozen in an isopentane. Isopentane was previously cooled down in the bucket of liquid nitrogen. Some samples were stored in -80°C; others were used for immediate cryostat sectioning.

4.2.2 Cryostat sectioning

Frozen samples were cut in 0,8μm sections using a cryostat and mounted on microscope slides. Slides were stored at - 80˚C until stained.

4.2.3 Fixation

All slides were fixed before other use. Fresh slides which were stained directly after cutting were fixed in 100% acetone for 10 minutes. Frozen slides were transferred directly from the freezer to 4˚C cold 50% acetone for 30 seconds and then to ice-cold 100% acetone for 5 minutes. Slides were air-dried at room temperature.

4.2.4 Hematoxylin and eosin staining

Fixed slides were dipped into hematoxylin for 3 minutes, rinsed in deionized water and then washed with tap water for 5 min. After these washing steps sections were rapidly dipped (8-12x) in acid ethanol and washed again, first with tap water (2 min) and then with deionised water (2 min). Before eosin staining was performed, excess water was blotted from the sections. Then sections were dipped in eosin for

30 seconds followed by dehydration. Sections were dehydrated first in 95% ethanol (for 3x5min), then in 100% ethanol (for 3x5min) and then in xylene (for 45min). Finally, the slides were mounted with xylene-based mounting medium and covered with a cover slip. Sections were dried overnight in the hood and visualised using a fluorescence microscope (Carl Zeiss) the next day.

4.2.5 Immunohistochemistry

To identify the distribution of immune cells within the tissue, T cells, B cells, macrophages, DCs and Neu were detected by immunohistochemistry. For this method primary antibodies (Ab) which bind to specific cells markers were used. Afterwards, this antigen-antibody interaction was visualised by adding a secondary Ab that binds to the primary Ab and that was labelled with a fluorescence dye. (Table.1)

Fixed slides were rehydrated in PBS for 5 minutes. Before rehydration each section was encircled with a DAKO pen. In order to block unspecific binding of secondary Ab, slides were incubated with 5% normal goat serum (NGS) in PBS for 30 minutes in a humidity chamber. When biotinylated Ab were used, endogenous biotin was blocked using a biotin blocking kit. After blocking, slides were incubated with primary Ab diluted in 1% NGS in PBS for one hour in humidity chamber, then washed twice in PBS for 5 minutes. This was followed by incubation with a secondary Ab diluted in 1% NGS in PBS for one hour in humidity chamber in the dark. After two final washing steps in PBS, slides were mounted with fluorescence mounting medium and visualised the following day with fluorescence microscope (Carl Zeiss).

Table 1. Antibodies for immunohistochemistry

Staining	Specific marker	Biotin block	Primary antibody	Dilution	Secondary antibody	Dilution	Isotype control	Dilution
T cells	CD4	No	rat-anti mouse CD4 (L3T4)	1:100	Alexa Flour 488 goat anti-rat IgG	1:1000	Purified rat IgG2α,κ	1:100
Neu	Ly6G	No	Purified anti-mouse Ly-6G 1A8	1:100	Alexa Flour 488 goat anti-rat IgG	1:500	Purified rat IgG2α,κ	1:100
Mφ	F4/80	No	F480	1:100	Alexa Flour 488 goat anti-rat IgG	1:400	Purified rat IgG2α,κ	1:100
B cells	$B220^{bio}$	Yes	Biotin anti-mouse CD45R/B220	1:500	Streptavidin, Alexa Fluor 594 conjugate	1:200	Biotin mouse anti-rat IgG2α	1:500
DC	$CD11c^{bio}$	Yes	Biotin anti-mouse CD11c (HL3)	1:100	Streptavidin, Alexa Fluor 594 conjugate	1:200	IgG hamster	1:100

4.2.6 Cell counting

The number of immune cells per 1000 lamina propria cells was counted separately for small intestine, proximal and distal colon using the ImageJ software.

5 Results

5.1 Mice description

Earlier reports showed that Muc2$^{-/-}$ mice tend to develop spontaneous colitis. It was observed that these mice also showed growth retardation and weight loss. Analyses by flow cytometry showed that in lamina propria cells in some Muc2$^{-/-}$ mice an increased percentage of Neu could be found. These mice had an overall increase of cells compared to C57BL/6 wild type (WT) as well as Muc2$^{+/-}$ mice (control) and thicker colon walls. In this study we wanted to analyse different cells of the immune system, *in situ*. For these experiments three Muc2$^{-/-}$ (M1, M2, M3), two control (C1, C2) and three WT (WT1, WT2, WT3) mice were used. All three Muc2$^{-/-}$ mice showed obvious signs of intestinal inflammation at the time of sacrifice. They had soft stool, enlarged rectum, swollen and prolapsed colon. In the most inflamed mouse (M1) the colon was almost twice as big compared to control mice and the thickness of the intestinal wall was increased in the middle part. Interestingly, the small intestine of this mouse was smaller than the others. Otherwise no size differences between the small intestines of the rest of the mice were observed. None of the control or WT animals showed such symptoms.

5.2 Histologic characterisation of colon and small intestine

The histology of colon and small intestine of Muc2$^{-/-}$ mice were compared to WT or control mice by staining tissue sections of the respective tissues with hematoxylin and counterstained then with eosin (H&E staining). Hematoxylin stains nuclei of cells in purple and eosin stains proteins in pink. In result in our samples nuclei are stained purple, whereas cytoplasm and extracellular matrix are stained with varying degrees of pink. This enables us to see the basic histological structure of the tissue.

For this staining we used half "Swiss rolls" slides, thus we were able to observe the histology of whole colon and small intestine and catch eventual changes between proximal and distal part. To prepare "Swiss rolls" colon and small intestine were cut longitudinally and rolled up using a toothpick, so that the distal part of the tissue was in the centre and the proximal part outside. Tissue samples were snap frozen and sectioned afterwards.

One of the most obvious observations was the enlarged colon in knockout compared to both control and WT animals. When analysing the micrographs of the H&E stained samples the crypts in the colon of Muc2$^{-/-}$ animal were found to be enlarged and prolonged; often the architecture was lost (Fig.1D). These changes occurred mainly in the distal part but in the most inflamed animal (M1, M2) we could see these changes also more proximally.

Our analyses of the small intestine did not reveal any morphological difference between the three groups except M1 mouse where small intestine was bit smaller. Although the structure of crypts and villi was preserved in all tissue samples, on some samples from Muc2$^{-/-}$ mice we also observed proliferation of villi. (Fig.1A, B)

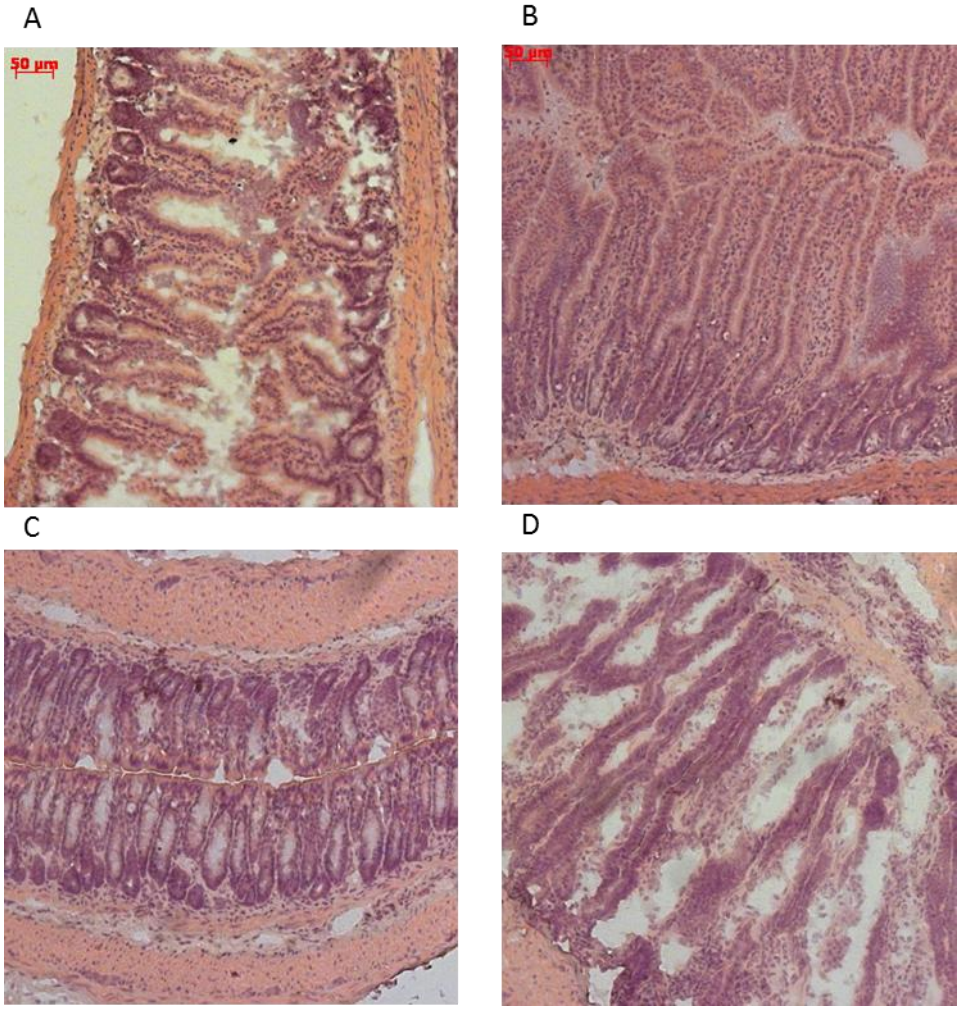

Figure 1 Histological characterisation of small intestine (A, B) and colon (C, D).
Tissues section of "Swiss rolls" from colon and small intestine of Muc2$^{-/-}$ and control mice were stained using H&E. Hematoxylin stains nuclei purple, eosin cytoplasm in various degrees of pink. The representative pictures of small intestine of control (A) and Muc2$^{-/-}$ (B) and colon of control (C) and Muc2$^{-/-}$ mouse (D) are shown. Original magnification: 100x.

5.3 Immunohistochemistry and titration of antibodies

In order to analyse different cell populations *in situ* the method had to be established. Therefore, different specific target epitopes were chosen enabling an identification of Neu via Ly6G, monocytes / macrophages were stained for with F4/80, dendritic cells were identified by using CD11c, T cells (Tc) with an Ab against CD4 and B cells (Bc) by detecting B220 on the cells surface. Then we focused on which particular clone of primary Ab and appropriate secondary Ab would give us the best signal. (Tab. 2) Finally, we titrated the concentration of selected Ab.

Table 2. Antibodies for immunohistochemistry

Cell type	Surface marker	Primary antibody	Dilution	Secondary antibody	Dilution	Result
Neu	Ly6G	Purified Ly6G (1A8)	1:100	Goat anti rat^{488}	1:500	+
		Biotinylated Ly6G	1:100	Streptavidin, Alexa Fluor 594 conjugate	1:200	-
MØ	F4/80	Purified F4/80	1:100	Goat anti rat^{488}	1:400	+
DC	CD11c	CD11c (N418)	1:100	Goat anti hamster	1:400	-
		Biotinylated CD11c (HL3)	1:100	Streptavidin, Alexa Fluor 594 conjugate	1:200	+
		Biotinylated CD11c (N418)	1:100	Streptavidin, Alexa Fluor 594 conjugate	1:200	-
Tc	CD3	Biotinylated CD3 (145CII)	1:100	Streptavidin, Alexa Fluor 594 conjugate	1:200	-
	TCRαβ	Biotinylated TCRαβ	1:100	Streptavidin, Alexa Fluor 594 conjugate	1:200	-
	CD4	CD4 (GK1)	1:100	Goat anti rat^{488}	1:500	-
		CD4 (LT3)	1:100	Goat anti rat^{488}	1:1000	+
Bc	B220	Purified B220	1:100	Goat anti rat^{488}	1:500	-
		Biotinylated B220	1:500	Streptavidin, Alexa Fluor 594 conjugate	1:200	+

5.4 Increased number of neutrophils in distal colon of Muc2$^{-/-}$ mice

In previous experiments the concentration of Neu in intestinal tissue was set up as a marker of intestinal inflammation. Therefore, we first wanted to assess the distribution of Neu in colon of Muc2$^{-/-}$ mice compared to control and WT mice. For this we stained half "Swiss rolls" tissue sections from colon with anti-Ly6G Ab and later visualised them by the fluorescence microscope. We focused on the distribution of Neu throughout the colon and differences between proximal and distal part.

In colon from control and WT animals only few Neu scattered in the LP throughout the colon with no significant increase from proximal to distal part (Fig.3A, B). We found that often there were few cells in close association to lymphoid follicles and the rest of the LP was almost without any Neu. In contrast in Muc2$^{-/-}$ mice an increasing number of Neu accumulated in the LP. Even though in the proximal part of the colon more Neu were detected, a further increase could be seen in the distal colon (Fig.3C, D). Nevertheless, the biggest infiltration was not always found in the most distal part. In case of M1 the strongest infiltration of Neu occurred in the proximal part of the colon at the thickest part of the intestinal wall. However the infiltration of Neu in the distal colon of this animal was extremely high as well.

Next, we looked for Neu in small intestine tissue section from the same mice using the same staining protocol. Unlike colon, the distribution of Neu in the small intestine was very similar in both Muc2$^{-/-}$ and control mice. Only in the tissue of one control mouse (C2) we detected higher number of Neu on few places. Generally, more Neu were scattered in small intestine LP than in colon of control mice (Fig.3E, F).

Using ImageJ software the amount of Neu was assessed in 1000 LP cells, keeping small intestine, proximal and distal sections from the same animal separate. In colon no significant difference between control and WT mice could be determined (Fig. 2A, B). In the distal colon there was a big increase in the number of Neu in the two most inflamed Muc2$^{-/-}$ mice (M1, M3) and slightly higher number of Neu in less inflamed mouse (M2) compared to control and WT (Fig.2B). In the proximal part the situation was similar. In knockout mice the amount of Neu was higher than in control and WT mice and in the M1 mouse was even higher than in the distal part (Fig. 2A). No significant difference in the amount of Neu between knockout and control mice in small

intestine tissues was detected. Moreover, in contrast to colon tissues, control animals showed higher amounts of Ly6G positive cells than knockout animals (Fig. 2C).

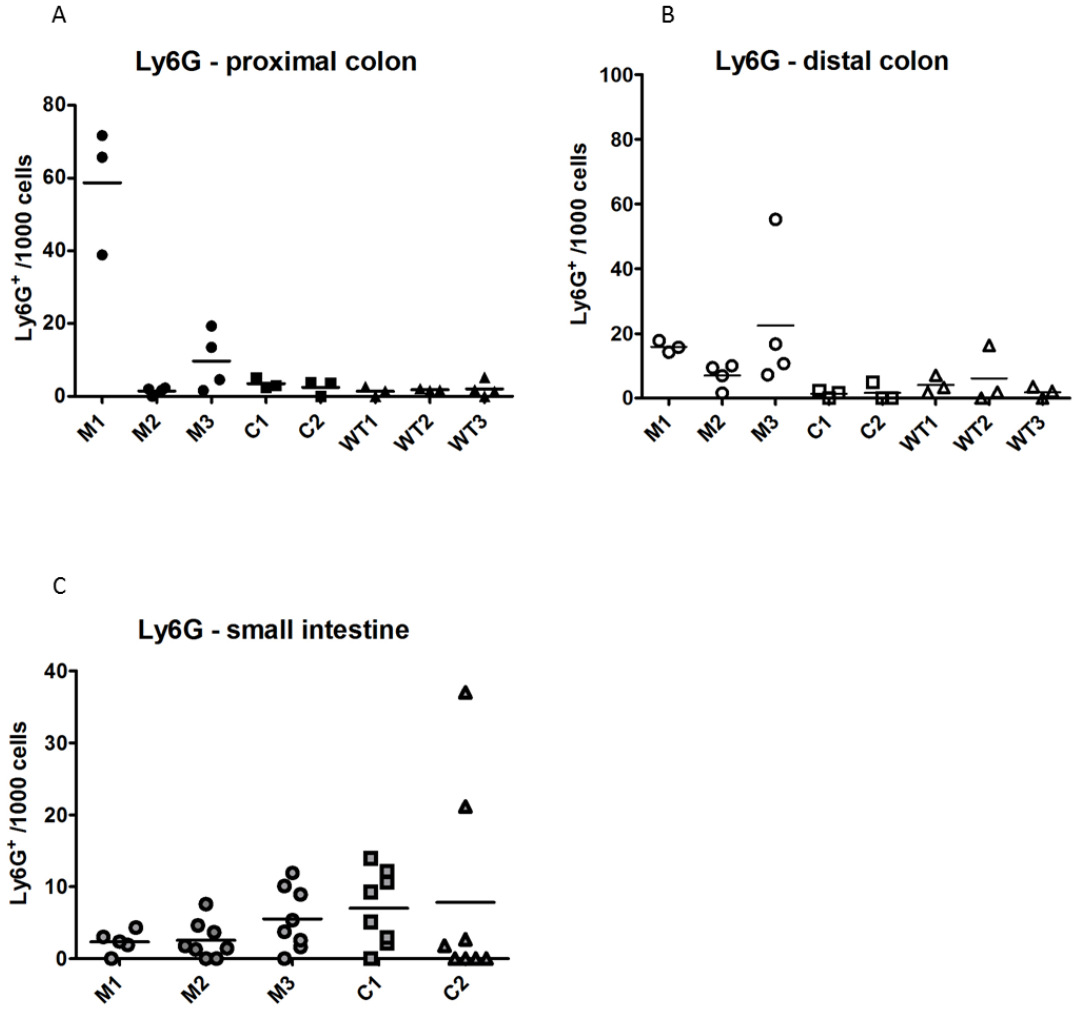

Figure 2 Number of Ly6G$^+$ cells in colon and small intestine
Aproximate amount of Neu per 1000 LP cells in small intestine (C), proximal (A) and distal colon (B) are shown. Tissue sections from Muc2$^{-/-}$ (M1- M3), control (C1-C2) and WT (WT1-WT3) mice were stained with anti-Ly6G and the number of Ly6G$^+$ cells per 1000 LP cells was counted. **A, B)** For each mouse we counted 3 representative images from proximal and distal colon. The values are given as means and each symbol represents one counting **C)** For each mouse we counted 8 representative images from small intestine – with exception of M1. Because of sample limitation only 5 pictures could be taken. WT mice were not included into this experiment. The values are given as means and each symbol represents one counting.

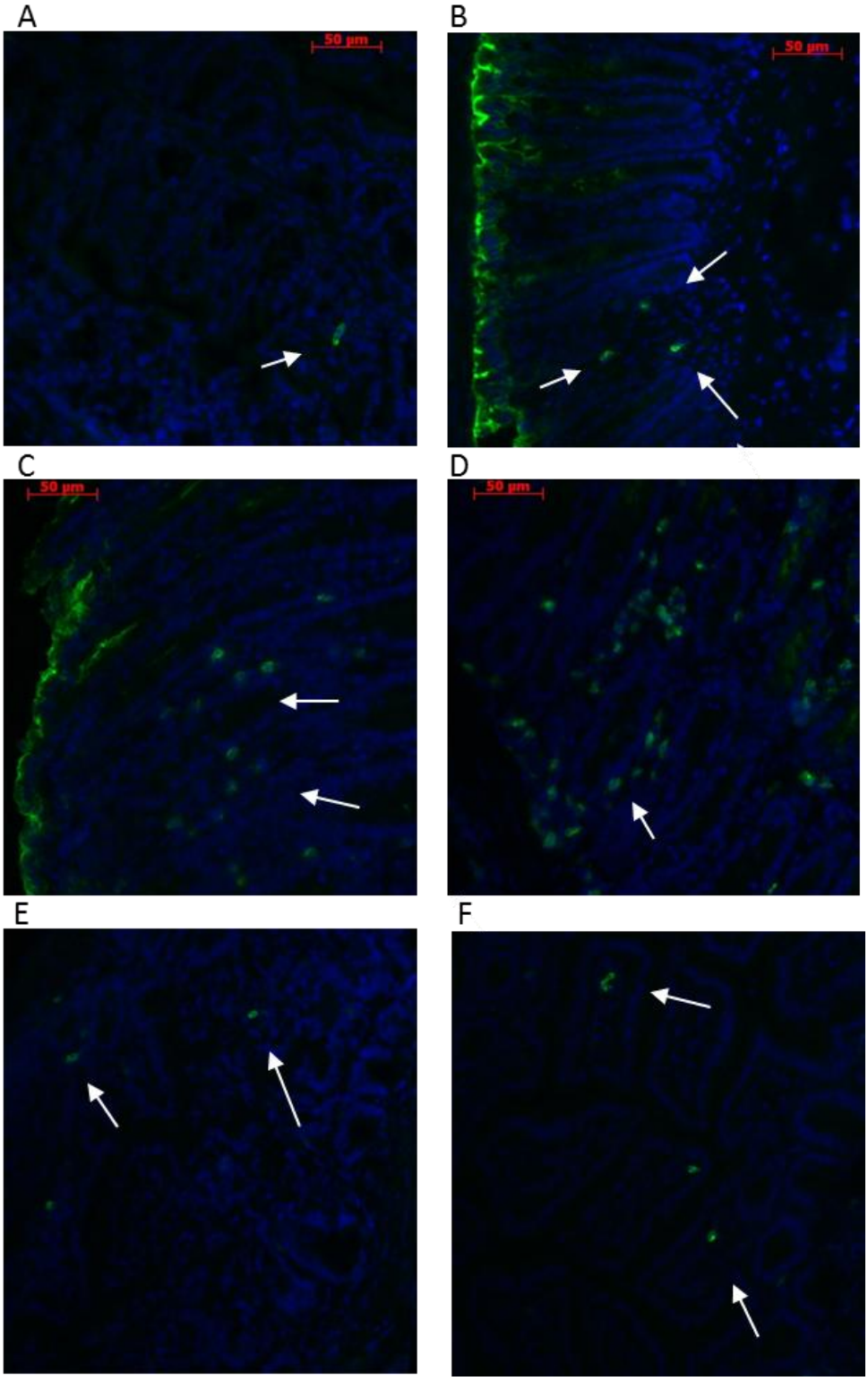

Figure 3 Neutrophil infiltration in the colon and small intestine
Immunohistochemistry analysis of colon (A, B, C, D) and small intestine (E, F) is shown. Tissue section of "Swiss rolls" from colon and small intestine of $Muc2^{-/-}$, control and WT mice were stained with anti-Ly6G (green) to detect Neu and counterstained with DAPI (blue) to indicate nuclei. Images show distribution of Neu (white arrows) in colon of control mice – proximal (A) and distal part (B), colon of knockout mice - proximal (C) and distal part (D) and small intestine of control mice (E) and knockout mice (F). Original magnification: 200x

5.5 Increase amount of macrophages in the distal colon of knockout mice

Next we looked at the distribution of macrophages in colon and small intestine. We used the same method as for Neu – immunohistochemistry analyses. Thus we stained tissue section with anti-F4/80 and analysed them by fluorescence microscope. We looked at the distribution of macrophages in proximal compared to distal colon as well as in small intestine.

Generally, macrophages were abundantly spread in LP of both tissues creating a dense network with their long processes. In colon, we observed greater infiltration of these cells in knockout mice, especially distally accumulating at specific sites (Fig.4). In addition there was huge amount of them in places with morphological deviations, as for example at the wall thickening in proximal colon of M1 mouse. In small intestine we could not detect any significant differences between control and $Muc2^{-/-}$ mice in the amount of macrophages (Fig.4E, F).

As we assessed the approximate numbers of macrophages in 1000 LP cells, we found that the amount of macrophages in colon was slightly higher in distal part than in proximal independent of the mouse genotype, the only exception was found in mouse C1. $Muc2^{-/-}$ mice had always higher numbers of macrophages than control and WT. This was notable even in the proximal colon, however the differences were not as obvious. The most macrophages were detected in distal colon of M1 mouse (Fig.5B). Unlike in colon we could not find any significant differences in numbers of macrophages in the small intestine (Fig.5C)

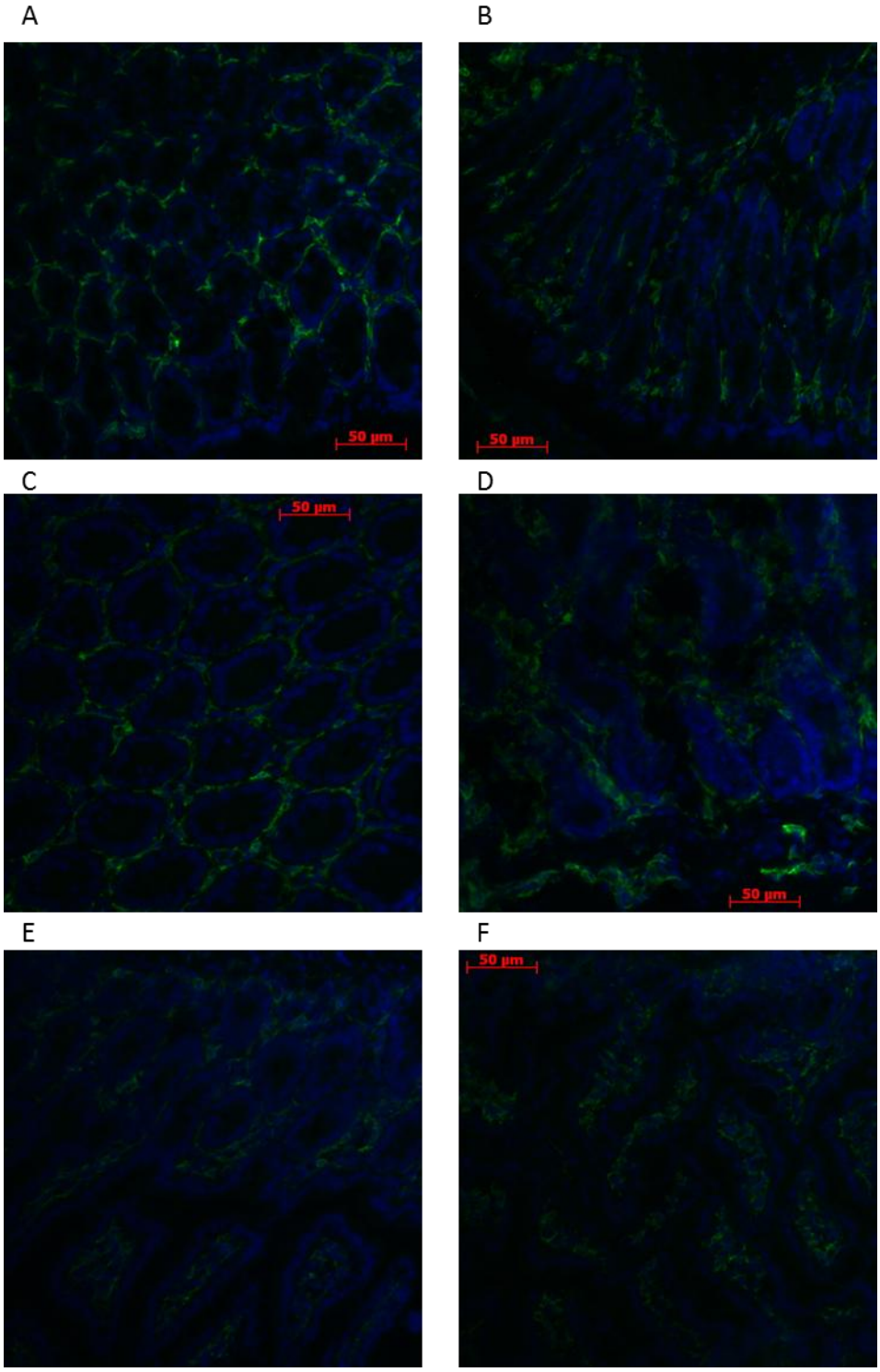

Figure 4 Distribution of macrophages in colon and small intestine
Immunohistochemistry analysis of colon (A, B, C, D) and small intestine (E, F) is shown. Tissue sections of "Swiss rolls" from colon and small intestine of $Muc2^{-/-}$, control and WT mice were stained with anti-F4/80 (green) to visualise macrophages and counterstained with DAPI (blue) to indicate nuclei. Images show distribution of macrophages in colon of control mice – proximal (A) and distal part (B), colon of knockout mice - proximal (C) and distal part (D) and small intestine of control mice (E) and knockout mice (F). Original magnification: 200x.

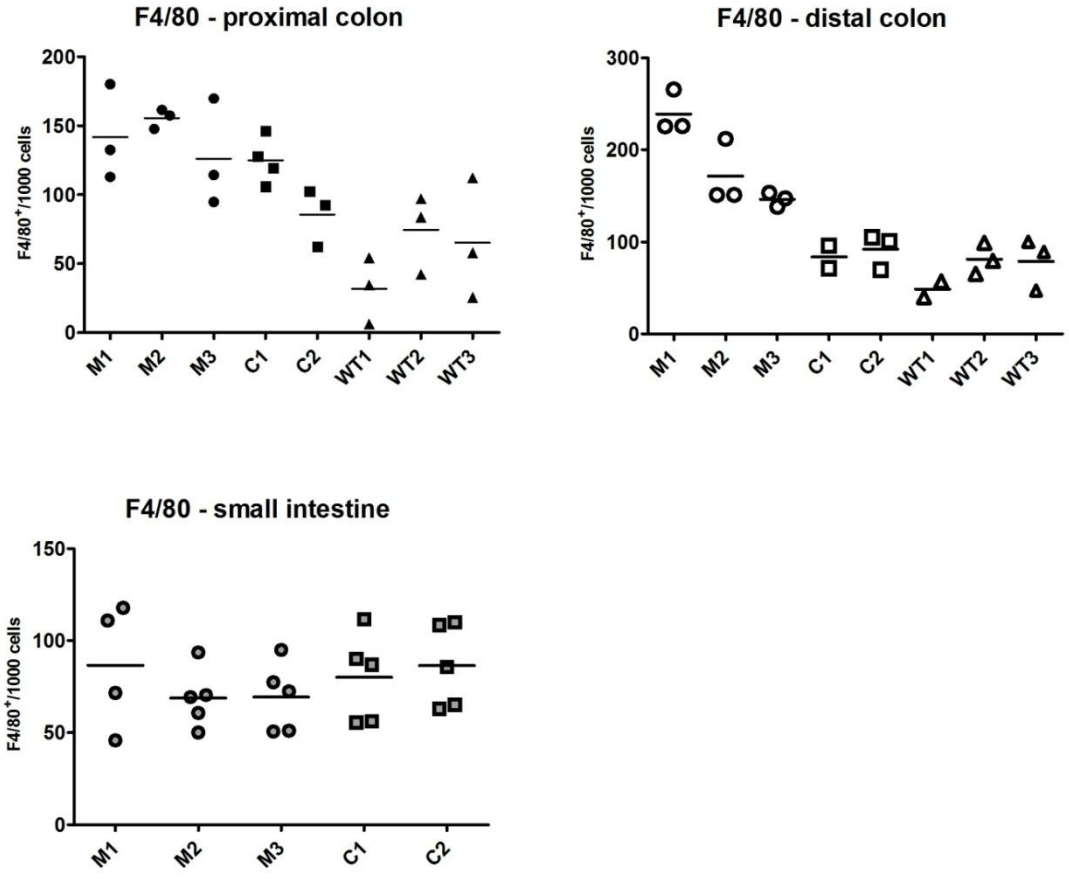

Figure 5 Amount of F4/80^{+} cells in colon (A, B) and small intestine (C).
Tissue sections from Muc2$^{-/-}$ (M1- M3), control (C1-C2) and WT (WT1-WT2) mice were stained with anti-F4/80. Using ImageJ the number of F4/80^{+} cells per 1000 cells in the LP of small intestine (C), proximal (A) and distal (B) colon were determined. WT mouse were not included in the analyses of small intestine. The values are given as means and each symbol represents one counting.

5.6 No significant diference in the distribution of DCs

Next we wanted to analyse the distribution of DCs in proximal and distal colon and small intestine of Muc2$^{-/-}$ mice compared to control and WT mice. The immunohistochemistry analyses with anti-CD11c showed that in all our tissues CD11^{+} cells – representing DCs – were abundantly present in follicles and in small intestine in PPs. Moreover, DCs were scattered in LP, sometimes forming small clusters (Fig.7). When we determined the amount of DCs per 1000 cells the numbers varied – especially in small intestine and no significant differences in the distribution between neither colon nor small intestine were found (Fig.6).

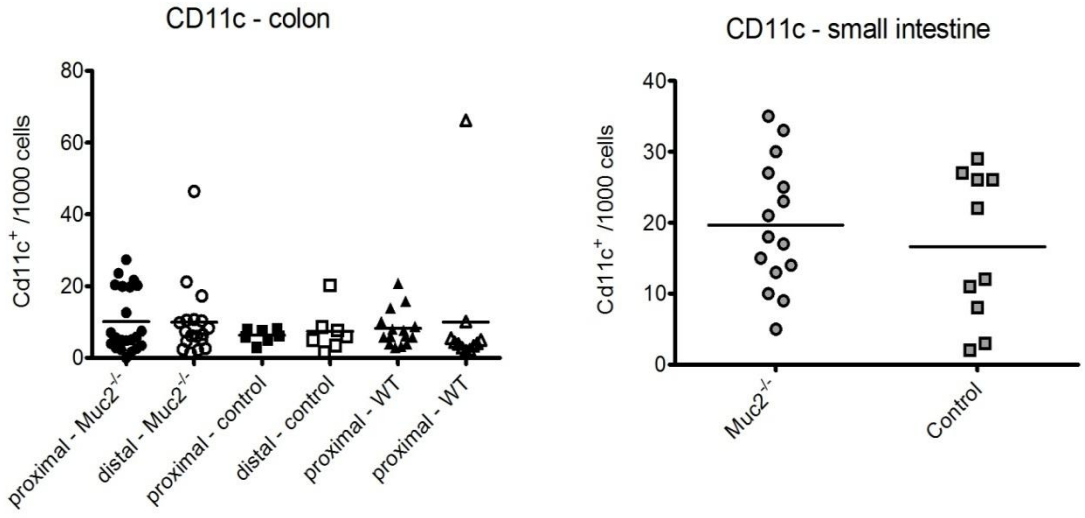

Figure 6.No alterations in the distribution of intestinal DCs.
Amount of CD11c^{+} cells per 1000 cells in the LP of small intestine (B), proximal and distal colon (A) is shown Tissue sections from Muc2$^{-/-}$, control and WT mice were stained with anti-CD11c. Using ImageJ the number of CD11c^{+} cells per 1000 cells in the LP of small intestine (C), proximal(A) and distal (B) colon were determined. Results from mice from the same groups (Muc2$^{-/-}$, control, WT) were put together. WT mouse were not included in the analyses of small intestine. The values are given as means and each symbol represents one counting.

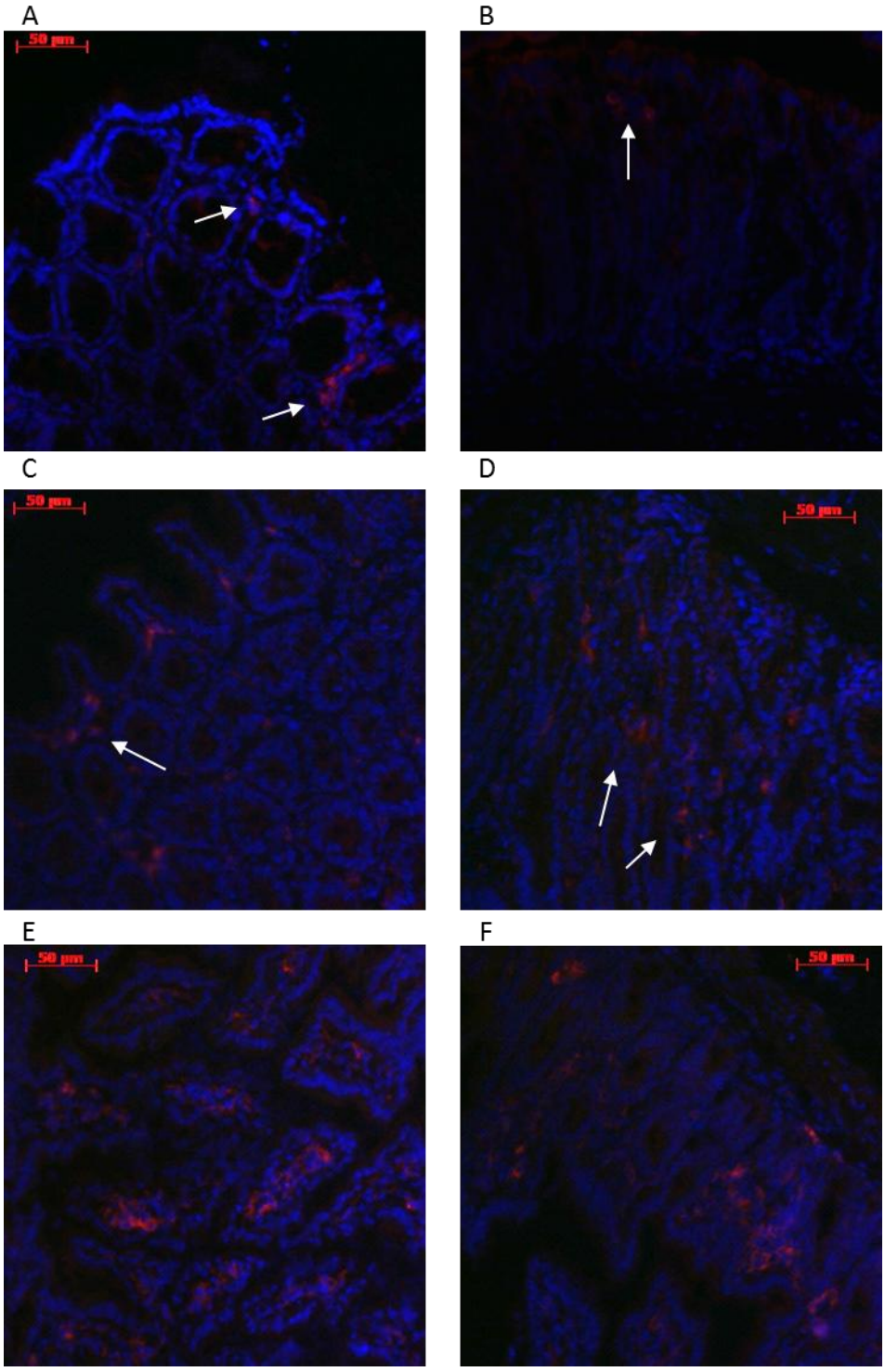

Figure 7 Distribution of Dc in colon and small intestine
Immunohistochemistry analysis of colon (A, B, C, D) and small intestine (E, F) is shown. Tissue sections of "Swiss rolls" from colon and small intestine of $Muc2^{-/-}$, control and WT mice were stained with anti-CD11c (red) to visualise DC and counterstained with DAPI (blue) to indicate nuclei. Images show distribution of DC (white arrows) in colon of control mice – proximal (A) and distal part (B), colon of knockout mice - proximal (C) and distal part (D) and small intestine of control mice (E) and knockout mice (F). Magnification: 200x

5.7 Increased number of T cells ($CD4^+$) in inflamed mice

Next, we analysed the distribution $CD4^+$ T cells in the colon and small intestine of $Muc2^{-/-}$ compared to control and WT mice and focused on changes in the amount of these cells throughout the intestine. To visualize T cells we stained half "Swiss rolls" tissue sections from $Muc2^{-/-}$, control and WT mice with anti-CD4 Ab. We determined the amount of $CD4^+$ T cell on representative micrographs from each mouse using ImageJ software.

In general, these cells were quite abundantly spread throughout LP of both tissues and many were located in follicles, respectively PPs in small intestine. On some places they were grouped together forming small clusters. In colon we found increasing numbers of $CD4^+$ T cells in $Muc2^{-/-}$ compared to both control and WT animals, while in small intestine we did not (Fig. 8).

This observations were supported with the evaluation of $CD4^+$ T cells in 1000 LP cells. In colon of $Muc2^{-/-}$ mice the $CD4^+$ T cells in LP were much more abundant compared to control and WT mice and the number was even greater in the distal part. However we did not observe any major differences in the distribution of these cells between the proximal and distal part in control and WT mice (Fig.9A, B). In small intestine the distribution of $CD4^+$ cells was more or less the same in all samples (Fig.9C). Because of lack of the tissue, we could not include M1 mouse into this experiment.

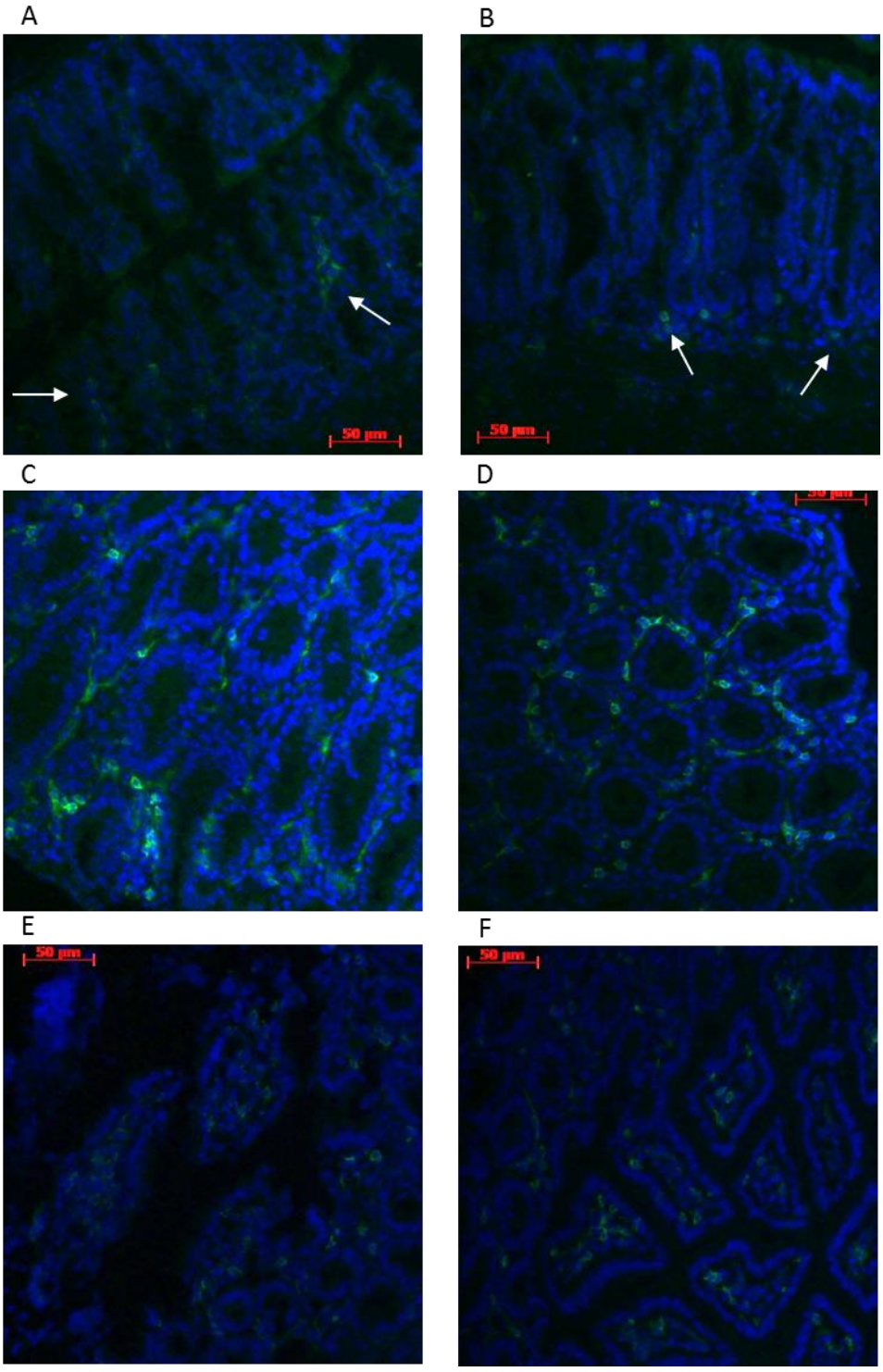

Figure 8 Distribution of $CD4^+$ T cells in colon and small intestine
Immunohistochemistry analysis of colon (A, B, C, D) and small intestine (E, F) is shown. Tissue section of "Swiss rolls" from colon and small intestine of $Muc2^{-/-}$, control and WT mice were stained with anti-CD4 (green) to visualise $CD4^+$ T cells and counterstained with DAPI (blue) to indicate nuclei. Images show distribution of $CD4^+$ T cells (white arrows) in colon of control mice – proximal (A) and distal part (B), colon of knockout mice - proximal (C) and distal part (D) and small intestine of control mice (E) and knockout mice (F). Original magnification: 200x

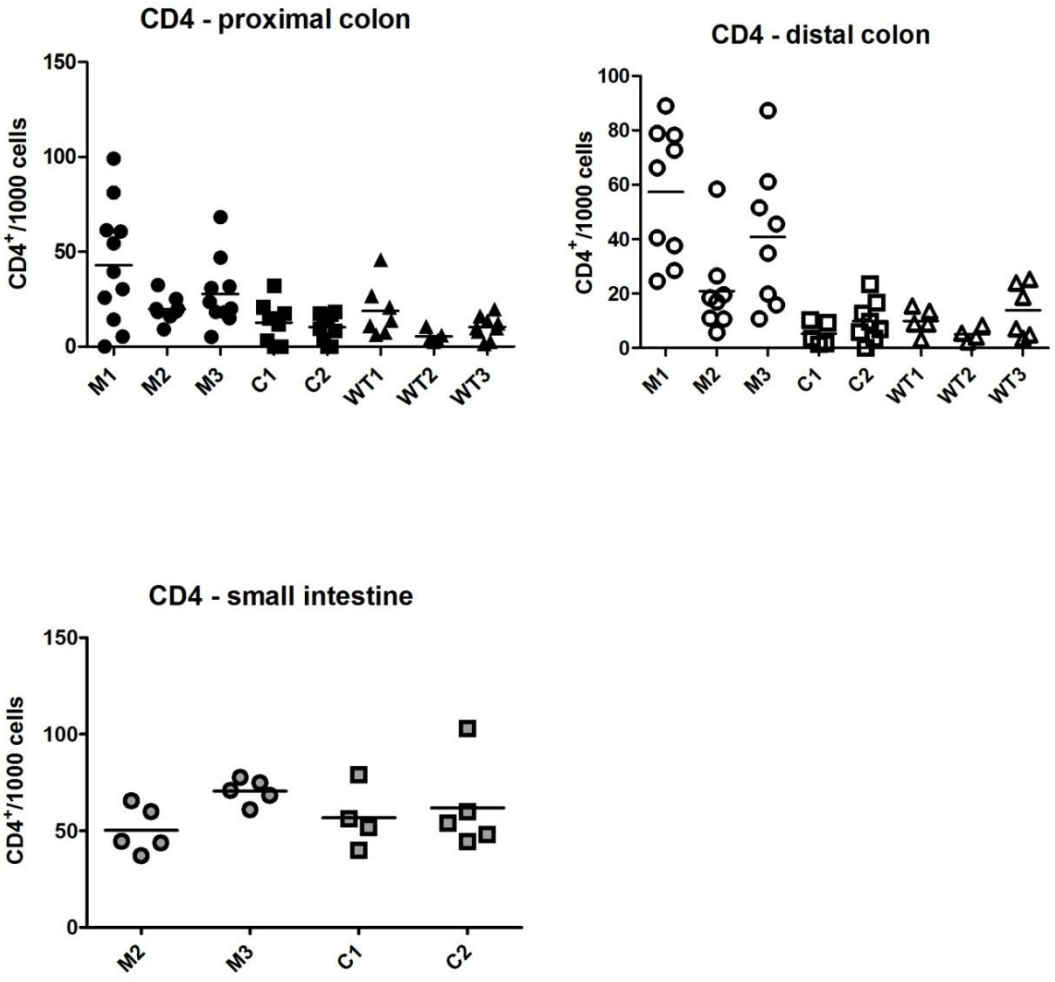

Figure 9 Amount of $CD4^+$ cells in the colon (A, B) and small intestine (C)
Tissue sections from $Muc2^{-/-}$ (M1- M3), control (C1-C2) and WT (WT1-WT2) mice were stained with anti-CD4. Using ImageJ the number of $CD4^+$ T cells per 1000 cells in the LP of small intestine (C), proximal (A) and distal (B) colon were determined. WT mouse were not included in the analyses of small intestine. The values are given as means and each symbol represents one counting. Because of lack of the tissue, we could not include mouse M1 in this experiment.

5.8 Increase of lamina propria B cells in the distal colon and small intestine of Muc2$^{-/-}$ mice

Finally, we stained tissue sections with anti-B220 Ab to analyse the localisation of B cells/plasma cells *in situ* in both small intestine and colon tissues of Muc2$^{-/-}$ and appropriate control animals.

In the healthy intestinal tissue B cells are homed to the PPs of small intestine, respective follicles in the colon. Similarly we observed this phenomenon in tissues of all groups of our experimental mice. In colon of control and WT mice most of the B cell were found in the follicles and just very few of them were scattered in the LP throughout whole colon. In contrast in knockout animals, we found more B cells scattered in the LP and the amount increased from proximal to the distal part, especially in M1 and M3 mouse (Fig.10). In proximal colon evaluating the amount of B cells distributed in LP, no significant difference between Muc2$^{-/-}$, control and WT animals were detected, while in the distal colon significantly higher amounts of B cells were identified by fluorescence microscopy compared to distal colon of all control and WT mice. Moreover a significant increase compared to proximal colon of same mouse was detected. (Fig. 11A)

At the first sight, in small intestine the distribution of B cells seemed to be similar in both groups, Muc2$^{-/-}$ and control. Nevertheless results from counting showed that the number of B cells scattered outside follicles was significantly higher in knockout mice than in control mice. However, the absolute number of B220^{+} cells in small intestine was lower compared to colon samples (Fig.11B). Small intestine of WT mice was not included in this experiment because of lack of material.

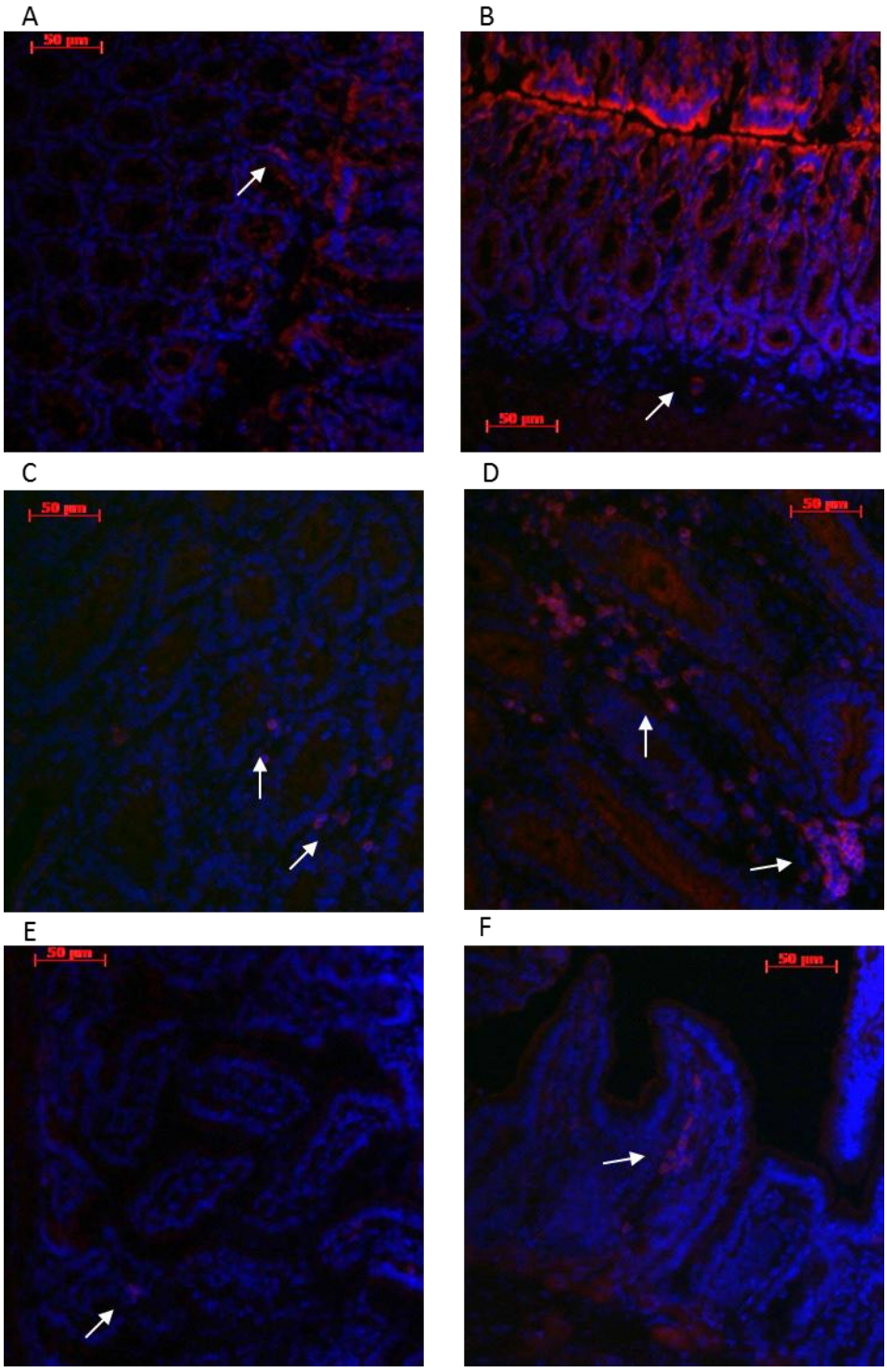

Figure 10 Increase number of B cell in the distal colon and small intestine of $Muc2^{-/-}$ mice
Immunohistochemistry analysis of colon (A, B, C, D) and small intestine (E, F) is shown. Tissue section of "Swiss rolls" from colon and small intestine of $Muc2^{-/-}$, control and WT mice were stained with anti-B220 (red) to visualise B cells and counterstained with DAPI (blue) to indicate nuclei. Images show distribution of B cells (white arrows) in colon of control mice – proximal (A) and distal part (B), colon of knockout mice - proximal (C) and distal part (D) and small intestine of control mice (E) and knockout mice (F). Original magnification: 200x

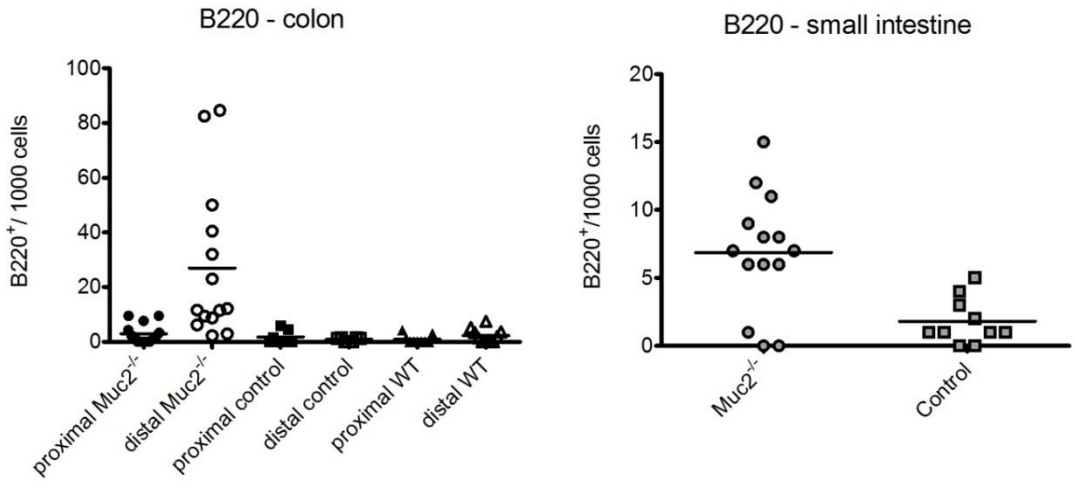

Figure 11.Increase number of B cells in the distal colon and small intestine of $Muc2^{-/-}$ mice
Amount of $B220^{+}$ cells per 1000 cells in the LP of small intestine (B), proximal and distal colon (A) is shown. Tissue sections from $Muc2^{-/-}$, control and WT mice were stained with anti-B220. Using ImageJ the number of $B220^{+}$ cells per 1000 cells in the LP of small intestine (C), proximal(A) and distal (B) colon were determined. Results from mice from the same groups ($Muc2^{-/-}$, control, WT) were put together. WT mouse were not included in the analyses of small intestine. The values are given as means and each symbol represents one counting.

6 Discussion

6.1 Overall aim of Muc2 project

$Muc2^{-/-}$ mice are genetically modified by replacing of the Muc2 gen and therefore their body is not able to produce Muc2 mucin. Lack of the main structural component of mucus results in general loss of the protective inner mucus layer, which under normal, healthy condition avoids direct contact of commensal bacteria with the intestinal epithelium. Thus, commensal bacteria, as well as any eventual pathogens, are in direct contact with the epithelial cell layer and can easily breach the epithelium (Johansson et al., 2008). Initial observations of $Muc2^{-/-}$ mice have shown that these mice develop spontaneous colitis and later on even colon cancer. (Van der Sluis et al., 2006). Because of this Muc2 mice are important experimental animal models to investigate the physiologic function of Muc2 as a part of mucus layer and to study the role of this epithelial barrier in pathology of inflammatory colitis and colorectal cancer.

It is believed that changes in quality and quantity of mucus may be a possible aetiological factor in pathogenesis of IBD. This complex disease is characterised by chronic gastrointestinal inflammation of parts of GIT. In patients with IBD among others alterations in secretory mucins have been described. Moreover, in many animal models the development of colitis can be prevented or attenuated if animals are kept in a germ-free environment and often the symptoms could be alleviated by antibiotic treatment (Sartor, 1997). This confirms that the presence of microbial factors may play a role in initiation and/or perpetuation of this disease.

The overall aim of Muc2 project is to characterize the immune status of the mucosal immune compartment of the small intestine and colon of $Muc2^{-/-}$ mice. Initial studies in our group have focused on the composition of the cells isolated from LP from colon and small intestine from $Muc2^{-/-}$ mice. It was shown that once the inflammation begins $Muc2^{-/-}$ mice have higher ratios of Neu compared to control or WT animals. Furthermore it was discovered that in tissues of inflamed $Muc2^{-/-}$ mice the up regulation of pro-inflammatory cytokines is not that significant but instead of that anti-inflammatory cytokines are decreased compared to control animals. This may indicate that the cause of the inflammation in these animals is rather the loss of tolerance than an activated immune response. To better understanding what is going on in the colon

and small intestine of Muc2$^{-/-}$ mice in this study we focused on *in situ* analyses of different immune cells within these tissues.

6.2 Differences in the histology and morphology of colon in inflamed Muc2$^{-/-}$ mice

In our experiments three knockout, two control and three WT mice were used. All these mice were between 20 - 25 weeks old. Other studies have shown that the Muc2$^{-/-}$ mice showed first clinical signs of colitis already at the age of 5 weeks and this signs aggravated as the mice aged (Van der Sluis et al., 2006). Because of the advanced age at the time of sacrifice all our knockout mice showed already severe signs of intestinal inflammation as diarrhoea, enlarged and prolapsed colon and rectal bleeding. Thus, at this age the differences between knockout and control or WT mice were more obvious and it was easier to describe them. On the other hand it would be very interesting to see when exactly the inflammation starts and which changes anticipate it. Therefore in the future other experiments including mice of younger age should be done.

In order to get a better and deeper insight into changes, which occur during intestinal inflammation in the mucosal compartment, we first addressed the histological characteristic of colon and small intestine of Muc2$^{-/-}$ mice compared to WT and control mice. H&E staining of "Swiss roll" tissue section from colon revealed significant differences between Muc2$^{-/-}$ and control mice. Compared to the control the colon of knockout animal was enlarged, the intestinal wall was much thicker, crypts were prolonged and often their architecture was lost. These changes were more obvious in the distal part. On the other hand no differences between control and WT were observed.

These results were expected and they corresponded with previous studies suggesting that distal part of colon is more affected by the inflammation (Van der Sluis et al, 2006). However, in case of animals involved in our experiments the inflammation was confined not only to the distal colon. We observed this phenomenon for example in M1 mouse. This mouse had extremely enlarged colon with much thicker intestinal wall compared to both control or WT animals but this thickening of intestinal wall was biggest in the proximal part and as later immunohistochemistry analyses confirmed, this

was the place of the most severe inflammation with the highest infiltration of Neu, macrophages and $CD4^+$ T cells.

When we compared histologic characteristic of small intestine of $Muc2^{-/-}$ and control mice our experiments did not reveal big differences in structure neither size of the tissue. Only in M1 mouse small intestine appeared to be a bit smaller compared to others. On the other hand on some tissue sections from knockout animals, similar as in colon, we observed proliferation of villi. Unfortunately we did not have enough tissue to make a clear conclusion and if this was in a consequence of the loss of the mucus and severe inflammation of the colon or not should be more investigated.

6.3 Immunohistochemistry

The *in situ* analysis of immune cells was performed by the immunohistochemistry method. Immunohistochemistry is the method based on the interaction between an antigen and an antibody. The Ab is labelled with fluorescence dye, which after exposure emits light of specific wave length that can be detected by fluorescence microscope. In order to enhance the signal, fluorescence dye labelled secondary Ab is often used. This secondary Ab binds to the primary Ab that recognizes the antigen.

One of the biggest advantages of immunohistochemistry analyses is that this method enables us to see the real distribution of cells within the tissue. Moreover, by simple counting of cells on micrographs we are able to determinate approximate amount of these cells in the tissue.

On the other hand, as any other method, immunohistochemistry has its limits and the interpretation of the micrographs is always subjective. We are able to use just one or two markers to determine the cell. Yet all immune cells have on their surface more than one specific marker and often these markers are shared for more different cell types. Therefore the choice of suitable antibody is very important for the future results.

Other problem is that often we have to handle with a lot of background on the micrographs, which is caused by unspecific binding of antibodies. Thus, when we used anti-Ly6G Ab to detect distribution of neutrophils in the tissue, on micrographs from colon we observed very strong green staining on the top of the epithelial cell layer. This staining was stronger in the distal then the proximal colon but we did not detect similar phenomenon in the small intestine.

Intestinal epithelial cell layer is not just single cell layer which separate intestinal lumen and the LP but has many important functions and express many different receptors. For example PPRs which recognise microbe associated molecular pattern and thus play important role in initiating of immune response. Moreover epithelial cells are able to secrete antimicrobial proteins such defensins and cathelicidins (Macpherson, 2009). All this could be reason of unspecific binding of the Ly6G Ab. Therefore we suggest that even though this unspecific signal was strongest in distal colon of knockout animals, this probably does not have any connection to the state of inflammation or lack of the mucus layer and it was rather an unspecific staining of the epithelial surface as we observed the similar pattern in control and WT mice.

6.4 In situ analyses of the distribution of immune cells in colon and small intestine

Many different cell types, including members of the innate and the adaptive immunity, play essential role in the immunity response during intestinal inflammation. Macrophages and neutrophils of the innate immune system represent the first line of defence against many common pathogens. These cells recognise pathogens by means of surface receptors (PRRs) that are able to recognize and bind common constituents of many bacterial surfaces and thus are able to efficiently eliminate pathogens without previous contact (Janeway, 2005).

Neu are immune cells of, which main function is to phagocyte pathogens. They normally circulate in the blood and enter the tissue only during an inflammation, when they are the first cells entering the affected tissue from the blood vessels (Janeway, 2005). In previous studies the correlation between increase number of LP Neu and signs of inflammation was observed. Therefore, in these experiments, the frequency of colonic LP Neu was set up as a marker of inflammation in the $Muc2^{-/-}$ mice. Animals with the frequency >0,5 % Neu were taken as inflamed and animals with frequency <0,5 % as non-inflamed. Additionally it was shown that the Neu frequency in LP isolated from distal part of colon was much higher than in the proximal colon (Hengst, 2010).

Similarly to these previous results, in our experiments, we observed higher ratio of Neu in $Muc2^{-/-}$ animals compared to control and WT. When we apply the same set up

with the Neu frequency all our knockout mice could be described as inflamed. In contrast all control and WT mice were according to our results non-inflamed. Only the WT2 mouse had the ratio of Neu around 0,6 % in distal colon and originally should be described as inflamed. But this was rather an outliner value than a significant increase due to the fact that immunohistochemistry is not one of the most accurate method for determination amount of cells in the tissue and the these numbers should be taken as relative.

Considering the differences between proximal and distal part of colon, the Neu infiltration in distal colon of knockout animals was much greater than in proximal, with exception of M1 mouse. In this case, the highest infiltration occurred on the place where the intestinal wall was thickest – and this was in proximal colon. This mouse appeared to be the most inflamed from all our mice and the number of Neu in distal colon was very high as well. When analysing the micrographs from this mouse, we observed the infiltration of Neu even into the intestinal lumen. However, one of the reasons could be that the architecture of the LP was lost and therefore it was difficult to orientate in tissue sample of this mouse. In contrast, control or WT mice have not shown such increase in amount of Neu and the number of them scattered throughout LP was very low in both proximal and distal colon.

The immunohistochemistry analyses of small intestine did not reveal any significant differences between $Muc2^{-/-}$ and control animal. We did not include the WT animals because we haven´t had any tissues samples from them.

Macrophages are another type of phagocytic cells. They circulate in the blood in immature form as monocytes and differentiate into mature macrophages first after entering the tissue. The number of macrophages in the tissue increases during inflammation. After engulfing of pathogens they release pro-inflammatory cytokines to attract other immune cell to the site of inflammation. Moreover, they are able to initiate adaptive immune response as they belong to APC and display ingested antigens of their surface and present them to T cells. (Abbas, Lichtmann 2005)

Macrophages are abundant in the intestinal LP but they differ in many aspects from that one in other tissues. Intestinal macrophages release less pro-inflammatory cytokines after activation and are not as good in presenting ingested antigens, as the expression of co-stimulatory molecules is low. On the other hand their phagocytic function remains unaffected (Smythies et al., 2005).

Immunohistochemistry analyses of distribution of macrophages in colon and small intestine of $Muc2^{-/-}$ mice revealed significant increase of these cells in the distal colon compared to control and WT mice. A similar phenomenon was found in proximal colon. However, the increase was not that significant. In contrast, any changes appeared in the small intestine and the ratio of macrophages was more than less the same in all groups of mice. Thus both the increase of macrophages together with increase of Neu indicates inflammation of the colon in knockout animals, which was usually more severe in the distal part.

The innate immunity provides immediate, nonspecific, broad protection against microorganism before previous contact, but it could be overcome by many pathogens and does not lead to immunological memory. The ability to recognise pathogens specifically and provide the protection against reinfection are the features of the adaptive immunity, which is initiated when an innate immune response fails to eliminate a new infection. However, the innate immune cells make a crucial contribution to initiation of adaptive immune response.

The cells specialized in presenting antigen to T cells and thus initiate the adaptive immune response are DCs. DCs are professional APC which mostly arise from the common myeloid progenitor but in some cases they could also arise from the lymphoid progenitor. They play crucial role in maintaining homeostasis within the intestine avoiding the potential overreaction against commensal bacteria while maintaining the capacity to eliminate the pathogens.

DCs in the intestinal mucosa continuously sample intraluminal antigens from both bacteria and food. Upon exposure to certain antigen they migrate to MLN and activate T cells which results in their clonal expansion and differentiation. The direction of differentiation into various regulatory and effector T cells subsets depends on the nature and the context of the presented antigen (Westendorf et al., 2010). T cell reactivity is determined during the initial stage of its activation by DCs and can either initiate host defence to pathogen or induce tolerance to food antigen and commensals. The important role play the anatomical compartments where immature DCs reside. It is believed that every individual tissue generates its own type of DCs and many different subsets of mucosal DC in PP´s, MLN and LP have been described.

Similar to macrophages intestinal DCs are unique in their functions and phenotype, which is rather anti-inflammatory than inflammatory. It was shown that LP DCs in the healthy intestinal tissue are characterised by low expression of co-

stimulatory cytokines and produce high levels of the anti-inflammatory cytokine IL-10 in contrast to DCs from MLN and spleen. (Strauch et al., 2010)

According to another studies (Strauch et. al, 2010) during colitis a dramatic increase in numbers of $CD11c^+$ DC could be detected within intestinal LP. Moreover LP-DCs in inflamed colonic tissue showed a mature phenotype with higher secretion of co-stimulatory molecules (CD80) and lower levels of IL-10 and tumor necrosis factor alfa. In contrast to these results our immunohistochemistry analyses of LP DCs in the colon and small intestine did not reveal any significant differences in the distribution neither amount of these cells in knockout compared to control or WT mice.

Here we may see one disadvantage of immunohistochemistry, because we cannot use more specific markers to detect distinct subset of DCs at once. Thus we were able to detect only a one big population of LP DCs and we are not able to describe the particular changes within the DCs subsets as we saw by previous experiments by Hengst, 2010. In her experiments the increase of some DCs subsets ($CD11^+$ and CD103/CD11b double positive DCs) in colon LP was observed. While the total number of DCs did not differ from control neither WT mice.

Cells from the lymphoid lineage – lymphocytes – play important role in the adaptive immunity. Lymphocytes – T cells and B cells - are the only cells which are antigen specific. When matured circulate these cells continuously between the blood and lymphoid tissue. On their surface they display unique receptors which recognize specific antigen. After encountering antigen lymphocytes differentiate into specialised effector cells or memory cells.

There are two main subsets of effector T cells. Cytotoxic T cells ($CD8^+$ T cells), which play important role in killing cells, infected by viruses and T helper (Th) cells ($CD4^+$ T cells). T helper cells may differentiate into more subsets, which differ by its function. Th1 activate other immune cells (B cells, macrophages) and Th2 cells are important for the immune response against extracellular parasites. Other type is regulatory T cells (Treg), which after antigen activation supress the immune response. Treg play crucial role in the homeostasis in the intestine as they protect the overreaction of the immune system to food antigens or commensals (Janeway, 2005).

Additionally to increase number of Neu and macrophages the immunohistochemistry analyses revealed an increase in amount of $CD4^+$ T cells in proximal and distal colon of the most inflamed $Muc2^{-/-}$ mice (M1, M3). In M2 mice the increase of $CD4^+$ T cells likewise the amount of Neu and macrophages was not that

high. Therefore we suggest that the amount of $CD4^+$ T cells in the intestinal tissue is a result of activated adaptive immune response and relate with the degree of inflammation and the amount of Neu and macrophages. For our analyses we used anti-CD4 Ab as the only marker of T cells and because of this we were not able to determine which particular subsets of $CD4^+$ T cells are involved in this process. Thus we got only the overall insight on the distribution of $CD4^+$ T cells in LP of inflamed Muc2 knockout animals.

Second type of lymphocytes – B cells – differentiates after activation into the Ab-producing plasma cells. The secreted IgA antibodies are produced by the help of intestinal DCs, which sample penetrated bacteria as well as that one from the intestinal lumen and induce B-cells differentiation.

In the healthy intestinal tissue B cells are homed to the PPs of small intestine, respective isolated lymphoid follicles in the colon. Similarly, we observed this phenomenon in tissues of all groups of our experimental mice. In colon of control and WT mice most of the B cell were found in lymphoid follicles and just very few of them were scattered in the LP throughout whole colon. In contrast in knockout animals, we found more B cells scattered in the LP and the amount increased from proximal to the distal part, especially in M1 and M3.

Interestingly, we found increased number of LP B cells in the small intestine from knockout mice. This result was unexpected because we suggested that small intestine of $Muc2^{-/-}$ mice is not affected by inflammatory process as we did not see any other differences in number of other immune cells. The increase of B cells and plasma cells could be one of a compensation mechanism to the absence of the mucus and higher stimulation by commensals. Unfortunately the number of mice involved in our experiments was not that great and in the future this experiment should be repeated and confirmed by other method, for example FACS staining.

To summarize all our experiments we found that there is a significant increase of Neu and macrophages in colonic LP from $Muc2^{-/-}$ mice compared to control and WT mice. Neu and macrophages are also known as inflammatory cells and their number in the tissue rapidly increases during inflammation, which correlates with the fact that these mice were obviously inflamed. Additionally to the increase of these innate immune cells we observed an increase of adaptive immune cells: Th cells, which stimulate other immune cells by producing pro-inflammatory cytokines and also B cells/ plasma cells, which indicate that the adaptive immune response has been activated.

Because in previous studies the changes were always more severe in the distal part of colon we decided to assess these parts separately. In our experiments the distal part was always more affected by the inflammation than the proximal and the increase of immune cells was higher. The most inflamed mouse M1 was the exception; here the place of the highest infiltration of Neu appeared in proximal colon on the place where the intestinal wall was thickest.

It is known that mucosal immune system is highly adapted to the presence of commensal bacteria. Nevertheless like pathogens, commensal bacteria express molecular patterns that can be detected by TLR and trigger the innate immune response. Yet under healthy condition can commensal bacteria induce intestinal B and T cells, this happen without triggering the inflammation and the massive neutrophil infiltrate that is characteristic for a pathogen infection. (Macpherson, Uhr, 2004).

However, in our experiment we observed many changes which are characteristic for the process of inflammation. The inflammation was usually more severe in the distal part of colon where the highest concentration of commensal bacteria is. Thus we may see that absence of protective mucus layer causes imbalance in the intestinal homeostasis where commensal bacteria may trigger a massive immune response similar to that one caused by pathogen. This may have severe consequences and finally, end with the development of colorectal cancer.

Another and the most widely used animal model for experimental colitis is dextran sodium sulphate (DSS) colitis model. In this case animals are orally challenged by DSS in the drinking water. The inflammation is observed already after 3-5 days but the cause of the inflammation has not been known for a long time. However in studies of Johansson et al. (2010) was shown that DSS cause alteration in the inner colonic mucus layer which result in decrease thickness and increase permeability and allow bacteria to quickly penetrate, reach the epithelial cells and trigger the inflammatory process. Here we may see some similarities with our $Muc2^{-/-}$ model which confirm the importance of the protective mucus for the intestinal homeostasis

7 Conclusion

The aim of this study was the *in situ* analyses of the immune status of colon and small intestine of $Muc2^{-/-}$ mice. For the detection of different immune cells the immunohistochemistry method was used. We focused on the detection of the innate immune cells: Neu, macrophages and DCs and also the adaptive immune cells: T cells and B cells within the intestinal LP while keeping the small intestine (ileum), proximal and distal colon separately.

To summarise our results in the distal colon of Muc2 knockout mice we found an increase number of Neu followed by increase of macrophages, $CD4^+$ T cells and B cells/plasma cells compared to control and WT animals. In the proximal colon there was a significant increase of Neu in the most inflamed mice and slightly higher amount of macrophages and $CD4^+$ T cells. The number of DCs was in both distal and proximal colon unaltered. In the small intestine we did not observe any changes with the exception of B cells. The number of B cells and plasma cells scattered in the LP of small intestine from $Muc2^{-/-}$ mice was significantly higher compared to control and WT mice.

These results indicate that the mucus layer forms essential barrier protecting underlying epithelium. Loss of this barrier in $Muc2^{-/-}$ leads to the disruption of the intestinal homeostasis and spontaneous inflammation of colon followed by massive increase of inflammatory immune cells, Neu and macrophages, which later on leads to activation of specific immune response concerning activation and differentiation of lymphocytes into effector cells. The distal part of colon was more inflamed and this could be due to the higher amount of commensals present in the colon because the number of commensals increases from proximal to distal colon. The analysis of the small intestine showed that this part of intestine is not that severe affected by the loss of mucus and should serve as a control. However if the increase in number of B cells scattered throughout the LP has any connection to absence of mucus should be further investigated.

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